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# **The Contribution of Newly Discovered and Emerging Viruses to Human Disease**

Dung Van Nguyen



A thesis submitted for the degree of Doctor of Philosophy

**The University of Edinburgh**

**2015**

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## **Declaration**

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I declare that the work recorded within this thesis was completed entirely by myself at the Roslin Institute of the University of Edinburgh between October 2012 and October 2015. In cases where others have made a contribution to the results obtained, this is clearly stated within the text. This work has not been submitted for any other degree or professional qualification.

Dung Van Nguyen

2015

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## Abstract

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According to the World Health Organization, over 200 infectious diseases in humans originate from animals (zoonoses), posing significant threats to human health. Zoonotic agents account for the majority of emerging and re-emerging pathogens. The human-animal interface has been recognised as an important risk factor that facilitates viruses to cross the species barrier and establish infection in humans. This indicates a need to perform surveillance of human populations who are at high risk of zoonotic infection due to their frequent contact with animals, together with the animals to which humans are exposed. The VIZIONS (Vietnam Initiative on Zoonotic Infections) has been conducted to directly respond to that need.

The large virus family *Picornaviridae* include known emerging pathogens that have major impacts on the economies and human and animal health (e.g. foot-and-mouth disease virus, hand foot and mouth disease virus). Some enteroviruses (EVs) and parechoviruses in this family have been shown to be able to infect both humans and animals while a number of new picornaviruses (new EV variants, cosaviruses, cardioviruses, hunniruses) with unknown pathogenicity and zoonotic potential have been discovered. This thesis, as part of VIZIONS, hopes to address the following gaps in our knowledge of such viruses in six genera (*Enterovirus*, *Parechovirus*, *Cosavirus*, *Cardiovirus*, *Kobuvirus* and *Hunnivirus*) of the family *Picornaviridae*:

- 1) The prevalence and genetic diversity of picornaviruses in studied samples
- 2) The epidemiology and disease association of the identified viruses
- 3) The overlaps (if any) of picornaviruses circulating in animals and humans
- 4) Possible animal sources of picornavirus infections in humans

In order to do that, over 2,000 faecal samples collected from a wide range of hosts (pigs, rats, bamboo rats, shrews, bats, chickens, ducks, boars, civets, porcupines, monkeys and humans) were screened for picornaviruses by nested PCR and real-time PCR assays. Detection frequencies varied between viruses and sample origins with kobuvirus as the most commonly detected virus, followed by EV, cardiovirus and hunnivirus. Parechovirus and cosavirus were not detected.

Comparison of detection frequencies of viruses infecting pigs revealed a disease (diarrhoea) association with porcine kobuvirus (PKV) but not EV infections. However, differences in PKV viral loads between diarrhoeic and non-diarrhoeic pigs were not statistically significant ( $p = 0.22$ ). In addition, the PKV VP1 sequences from the two pig categories were not phylogenetically distinct. EV VP1 sequences obtained from pigs and boars showed high genetic diversity with four previously known types and nine new types (EV-G8 to -G16). Analyses of complete genome sequences of two new EV types provided evidence for inter-type recombination with a putative breakpoint in the 2A coding region.

Similarly, study on samples from monkeys showed endemic infection of EV but no overlap with EV variants in humans was observed. The majority of EV detected in monkeys were novel with evidence for chimeric genomes and putative recombination breakpoints in the 2A region. New criteria for the classification of EV were additionally proposed.

Characterization by sequencing of VP4/VP2 and VP1 regions or complete genomes of picornaviruses in rats and bamboo rats also showed relatively high genetic diversity. While these viruses can infect different species of rats, they were again genetically different from viruses detected in the studied human populations.

In summary, studies in this thesis provide substantial new information on the prevalence, genetic diversity and disease association of picornaviruses in the studied populations. However, picornaviruses detected from animals were consistently separate from those found in humans, consistent with a relatively limited zoonotic potential of members of the virus family.

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## List of the published papers that are included in the thesis

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**Oral presentation at the 18<sup>th</sup> International Picornavirus meeting:** High rates of infection with novel enterovirus variants in wild populations of mandrills and other Old World Monkey species. Europic 9-14 March 2014, Blankenberge, Belgium (<https://kuleuvencongres.be/europic2014/program>).

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## Abbreviations

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BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	complementary deoxyribonucleic acid
Ct	threshold cycle
CV	coxsackievirus
DAF	decay accelerating factor
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EAV	equine arterivirus
EMCV	encephalomyocarditis virus
ENc	effective number of codons
ERAV	equine rhinitis A virus
ERBV	equine rhinitis B virus
EV	enterovirus
FMDV	foot and mouth disease virus
GI	gastrointestinal
HAV-cr1	Hepatitis A virus cellular receptor 1
HCoV	human coronavirus
HFMD	hand, foot and mouth disease
HIV	human immunodeficiency virus
HPeV	human parechovirus
HRV	human rhinovirus
HuV	hunnivirus
ICAM-1	intercellular adhesion molecule 1
ICTV	International Committee on the Taxonomy of Viruses
IPTG	isopropyl $\beta$ -D-1-thiogalactopyranoside
IRES	internal ribosomal entry site
kb	kilobase
LB	Luria Bertani



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LDLR	low density lipoprotein receptor
LV	Ljungan virus
MEGA	Molecular Evolutionary Genetics Analysis
MGPs	magnetic glass particles
ML	maximum likelihood
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NCBI	National Center for Biotechnology Information
NHPs	non-human primates
NJ	neighbour joining
nt	nucleotide
OUCRU	Oxford University Clinical Research Unit
OWMs	Old World Monkeys
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PhD	doctor of philosophy
PKV	porcine kobuvirus
PV	poliovirus
RF	recombinant form
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	rounds per minute
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SAFV	Saffold virus
SARS	severe acute respiratory syndrome
SOPs	standard operating procedures
SVDV	swine vesicular disease virus
TAE	Tris-acetate-EDTA
T <sub>m</sub>	melting temperature
TMEV	Theiler's murine encephalomyelitis virus
TRV	Thera virus

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UK	United Kingdom
US	United States
UTR	untranslated region
UV	Ultraviolet
VCAM-1	vascular cell adhesion molecular 1
VHEV	Vilyuisk human encephalomyelitis virus
VPg	viral protein genome linked
WHO	World Health Organisation
WIN	Winthrop

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# Chapter 1. Introduction

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## 1.1. Introduction

Picornaviruses, members of the large and highly genetically diverse virus family (*Picornaviridae*), are of major economic and medical importance. Picornaviruses can infect a wide range of human and animal hosts (Table 1.1). Although most infections are asymptomatic, some picornaviruses cause mild illnesses, whereas infection with other picornaviruses results in serious conditions of the central nervous system, heart, skeletal muscles, and liver (Yin-Murphy & Almond, 1996). Pathogens of clinical importance in humans and other animals include the emerging hand, foot, and mouth disease viruses, poliovirus, hepatitis A virus and foot and mouth disease virus.

A number of novel picornaviruses belonging to genera *Enterovirus*, *Parechovirus*, *Cosavirus*, *Cardiovirus*, *Kobuvirus*, *Hunnivirus*, etc. have recently been discovered and described (Knowles *et al.*, 2012) with unknown zoonotic potential and disease association. Our understanding of the global epidemiology, transmission dynamics and genetic diversity of both the emerging and newly discovered picornaviruses is also limited. This thesis carried out as part of the VIZIONS project (Vietnam Initiative on Zoonotic Infections) (Rabaa *et al.*, 2015) aims to address some of these gaps in our knowledge by examining viral diversity, distribution of types, molecular epidemiology (incidence, age and seasonal distribution), recent evolution and potential disease associations of viruses in the picornavirus genera mentioned above in the VIZIONS samples and monkey samples from Cameroon.

These investigations apply recent advances in molecular technologies for the effective surveillance and diagnostic screening of viruses. Deep sequencing which requires no prior known knowledge of sequences to be detected is considered as the most effective tool for novel virus discovery (Knox *et al.*, 2012). Its application has also helped elucidate microbiological aetiology of diseases of unknown origins (Kriesel *et al.*, 2012; Yozwiak *et al.*, 2012). The method, on the other hand, has disadvantages of high costs of instruments and supplies, as well as methodological and bioinformatic complexity (Isakov & Shomron, 2011; McElroy *et al.*, 2014). Furthermore, it is most

effective on samples collected from sterile sites such as cerebrospinal fluid, and samples which other potential causative agents have been excluded. Pre-identification of potential agents in clinical samples beyond current diagnostic screening is, therefore, useful for subsequent selection of samples for deep sequencing. Meanwhile, the much simpler molecular method of polymerase chain reaction (PCR), when using well-designed primer sets, can enable rapid "generic" protocols for detection of wide ranges of viruses.

## **1.2. Zoonoses**

### **1.2.1. Introduction**

The World Health Organization (WHO, 1959) defines zoonoses as diseases or infections that are naturally transmissible between vertebrate animals and humans. Among 1,407 human pathogen species, 816 (58%) were classified as zoonotic in a review by Woolhouse and Gowtage-Sequeria (2005). The review also showed zoonotic pathogens accounted for 73% (130/177) of emerging or reemerging species. The occurrence of zoonoses relies on the human-animal interface, which is the continuum of contacts between humans and animals, either directly or indirectly through their environments, or their products (Gortazar *et al.*, 2014; Reperant *et al.*, 2012). People who have frequent and close exposure to animals are therefore at high risk, as indicated by modes of transmission of zoonotic pathogens: 35% by direct contact, 61% by indirect contact, 22% by vectors (Woolhouse & Gowtage-Sequeria, 2005).

Throughout history, human beings have suffered severe losses from the emergence of many infectious diseases, particularly those caused by viruses. Until now, Spanish Influenza was probably the worst pandemic, which killed more than 20 million people during 1918 – 1919. More recently, HIV remains incurable since its initial emergence in the early 1980s, with approximately 2 million deaths worldwide each year (Kuiken *et al.*, 2003). Meanwhile, H5N1 has attracted considerable attention due to its ability to cause fatal human disease (Kerkhove, 2009) and raised public anxiety that the virus could acquire the ability to transmit efficiently between humans while retaining its high pathogenicity (Parrish *et al.* 2008). Understanding the underlying causes that

drive the emergence of viruses may help to develop strategies for infection control and prevention.

### **1.2.2. Factors underlying the emergence of zoonotic pathogens**

The emergence of a disease combines two elements: the introduction of the pathogen into the human population and its subsequent spread and maintenance within the population. There are a number of factors involved in the emergence of new viral diseases. In addition to virus evolution, environmental factors may also have important contributions. These together with host population determinants such as rapid growth of the world population and global transportation favour the establishment and rapid spread of viral diseases (Daszak *et al.*, 2000; Jones *et al.*, 2013; Morse, 2004; Özbal, 2010; Parrish *et al.*, 2008; Wang & Crameri, 2014).

#### **a) ENVIRONMENTAL FACTORS**

Environmental factors, especially changes in climate and land use, are often considered as the specific cause of emergence. These factors facilitate contact between humans and new natural reservoirs either by placing humans in close proximity to reservoir or by changing conditions that favour an increased population of the microbe or its natural host (Morse, 2004).

**Land use** is changed due to most of the economic development activities like agricultural development or water projects, urbanization and deforestation. Disturbance of habitats as a result of change in land use, in turn, may influence the breeding sites or the biodiversity of vectors or hosts (Patz *et al.*, 2008). There are strong evidences that emergence of new viral infections is associated with increased human-reservoir contact as a result of change in land use. A recent example is Venezuelan hemorrhagic fever (Salas *et al.*, 1991), a new disease caused by an arenavirus. The disease emerged primarily in settlers moving into areas of forest cleared for agriculture which was a favourable environment for the probable reservoir host, the cane mouse *Zygodontomys brevicauda* (Lederberg *et al.*, 2003).



**Climate change**

Climate change is of a growing concern as a factor in the viral emergence. There are three major ways whereby warming climate has damaging impacts on human health:

- (a) creating conditions advantageous for outbreaks of infectious diseases to occur,
- (b) increasing the potential for transmissions of vector-borne diseases which are facilitated by wide distribution of vectors and the exposure of millions of people to new diseases and health risks, and
- (c) hindering the future control of disease (Mahmood, 2012).

It results in the fact that viruses which used to be restricted to tropical areas may now spread to Europe and North America, for instance. In other words, emerging diseases can extend or reach new geographic areas due to climate change. In fact, this has happened and has been recorded. In 2007, chikungunya virus caused the first outbreak in Europe of a disease that had previously been found only in the tropical regions around Indian Ocean. Global warming has created conditions that make it easier for the tiger mosquito (*Aedes albopictus*), the vector of chikungunya, to be able to move north and occupy areas in Italy and spread the disease. Although this virus has not been reported to cause death in humans, the outbreak warns that other tropical diseases with more serious consequences can occur in the same way (Grazzini, 2008). Another example is the emergence of West Nile virus in the United States during the summers of 2002 and 2004, when the temperatures were above average, a condition required for efficient transmission of the virus (Reisen, 2006).

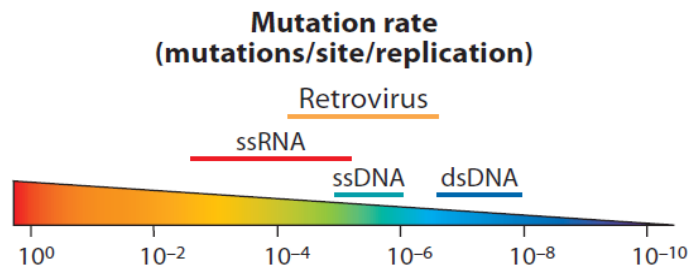
Similarly, deforestation and climate change are blamed for the emergence of Nipah virus in Malaysia, resulting in hundreds of reported cases of acute viral encephalitis in 1998 (Pike *et al.*, 2010). Fruit bats (*Pteropus vampyrus*, *Pteropus hypomelanus*) are thought to be the natural reservoir hosts of the virus (Chua *et al.*, 2002). The habitat loss due to forest fires and deforestation forced the fruit bats to search for fruits in orchards near the pig farms. The pigs in turn ate fruits contaminated with bat urine and saliva, and spread the virus to pig farmers (Jones *et al.*, 2013; Reperant *et al.*, 2012).

**b) VIRAL EVOLUTION**

In addition to the importance of ecological factors, it is also possible that genetic factors play an important role in the process of disease emergence (Holmes & Rambaut, 2004). Not all viruses require evolutionary changes to emerge in new hosts (Cutler *et al.*, 2010). In some cases of viral emergence, the evolution of the virus is necessary to allow efficient infection and transmission within the new host (Parrish *et al.*, 2008). Viruses can evolve quickly to adapt to the changing environmental conditions because they can acquire genetic variation by many mechanisms.

**Mutation**

The most common mechanism that affects all viruses is mutation which involves small changes in their genomic sequences. On average, a mutation happens during almost each replication cycle of virus genome (Holmes, 2009). Mutants can be point mutants (one base replaced by another) or insertion/deletion mutants. This can occur either in response to selective pressure and environmental conditions (such as radiation) or randomly as errors during genome replication for which RNA viruses generally have much higher mutation rates than DNA viruses do (Domingo, 2010). That is due to the inherent absence of RNA polymerase proofreading activity in RNA viruses, which leads to estimated mutation rates of  $10^{-3}$  to  $10^{-5}$  misincorporations per nucleotide copied (Domingo, 2010; Duffy *et al.*, 2008), while that corresponding figure for eukaryotes is just  $10^{-9}$  (Holmes, 2009). Although mutation has not been the mechanism leading to pandemic zoonotic influenza, it was of great concern when a controversial paper on avian influenza A/H5N1 mutant strain was published on Science (Herfst *et al.*, 2012) after debate. The authors showed just as few as five amino acid substitutions, introduced in the laboratory by site-directed mutagenesis and subsequent serial passage in ferrets, were sufficient for the avian influenza A/H5N1 strain to become airborne transmissible between these mammals. This finding highlighted the threat that highly pathogenic avian influenza A/H5N1 virus, which can cause morbidity and mortality in humans, may naturally acquire similar mutations to be transmitted by aerosol or respiratory droplet.



**Figure 1.1.** Comparison of mutation rates in viruses. Adapted from Duffy *et al.*, 2008.

## Recombination

Viruses can also achieve new genetic information by exchange of a short part between two strands of viral DNA/RNA during the process of viral nucleic acid replication inside the host cell, termed recombination, which can be of major evolutionary importance. In order for recombination to take place, a cell needs to be infected with at least two viruses. It occurs at dramatically different rates among viruses and is reported mainly from RNA viruses (Holmes, 2009). This mechanism is considered as a force to remove deleterious mutations or a means to create genomic diversity, whereby increasing the chance of finding a genetic combination that can adapt the new environment, such as a new host (Domingo, 2010; Holmes, 2011). For example, a number of poliomyelitis outbreaks were related to recombinant viruses between attenuated vaccine polioviruses and other circulating EVs (Adu *et al.*, 2007; Estívariz *et al.*, 2008; Kew *et al.*, 2002; Rakoto-Andrianarivelo *et al.*, 2008; Rousset *et al.*, 2003; Shimizu *et al.*, 2004; Yang *et al.*, 2003).

## Reassortment

The third most common mechanism of virus evolution is reassortment in which segments of genomes from different viruses infecting the same cell (mixed infections) are exchanged. Therefore, segment reassortment is a major evolutionary force which occurs only in some viruses with segmented genome and is a key mechanism for rapid novel virus creation (Holmes, 2011). Strains with novel reassortment can escape from the attack of adaptive immunity by introducing antigens to a naïve host population. New antigenic combinations acquired from recombination or reassortment may support emerging viruses in the process of cross-species jump to infect humans

(Domingo, 2010) or can create a novel virus that is both highly pathogenic and easily transmissible between humans (Özbal, 2010).

It is worth noting that many recent emerging viruses that cause human infections show active recombination or reassortment (Domingo, 2010). The continual change of genes that encode 2 envelope proteins of influenza A virus (haemagglutinin and neuraminidase) is a convincing example of how the virus has benefited from recombination and reassortment (Holmes, 2011). Reassortment which involved a genome of nonhuman origin is responsible for at least two human influenza pandemics in the 20<sup>th</sup> century. It was reinforced by the emergence of the H1N1 influenza (swine flu) in humans (Greenbaum *et al.*, 2012). First described in April 2009, the virus arose from a triple reassortment of genes from human, swine and avian influenza A viruses. Emerging in Mexico, this virus quickly spread the globe and was then declared the cause of a new pandemic by the World Health Organization (WHO). According to the recent WHO statistics (July 2010), the virus had killed more than 18,000 people since it appeared, however the real number from the H1N1 strain is "unquestionably higher" due to unconfirmed or unreported deaths (WHO, 2010).

### **c) TRAVEL**

Global travel has been considered as one of the major factors that enhance the risk of global spread of infectious diseases. Modern means of transportation like air transport facilitate the spread of infectious agents (Wilson, 1995). In 2005 Steve Fossett completed non-stop circumnavigation in a record time of just sixty seven hours by air while a century ago, the same journey took more than a year (Smith, 2009). Human migration facilitates the emergence of infectious diseases by unintentionally carrying pathogens or disease vectors in or on their bodies and luggage. Furthermore, their cultural traditions, habits and behavioral patterns may be not suitable for a new environment, increasing their risk for infection. A compelling example is the first severe infectious disease to emerge in the 21<sup>st</sup> century, severe acute respiratory syndrome or SARS, whose international spread was enabled by long distance travel (Ruan *et al.*, 2006). The rapid transmission of the virus, forced the WHO to issue emergency travel guidance and advice to airlines and travelers, providing case

definitions for probable and suspect cases of SARS (WHO, March 15, 2003). SARS was rapidly brought under control but had caused more than 8,000 confirmed cases and 774 deaths in 30 countries and regions (WHO, 8/15/2003) (Ruan *et al.*, 2006).

#### **d) DEMOGRAPHICS**

Animals have long been identified as reservoirs of zoonotic viruses and are responsible for most newly emerging viral infections in humans (Murphy, 1998; Taylor, 2001; Woolhouse & Gaunt, 2007). Meanwhile, the global human and livestock animal populations have undergone explosive growth. The world population reached more than seven billion people by the end of 2011, and it is projected to soar to over nine billion by 2050. Population growth has resulted in global overpopulation which favours infectious diseases by many ways, including increased travel, increases in wars and refugees, more overcrowded slums, environmental pollution and increasing interactions between humans and animals through hunting, trading of animal products, animal husbandry, and the domestication of animals or exotic pets. The probability of cross-species transmission, as a consequence, appears to be dramatically accelerating. For example, studies on the emergence of the H5N1 influenza viruses, which have caused sporadic outbreaks in Asia and Europe since 1997, have showed that direct contact with sick or dead poultry or wild birds is the most important risk factors for human H5N1 infection (Zhou *et al.*, 2009). HIV is another example. Human contact with wild primates through hunting and butchering in the early 20th century led to the introduction of simian immunodeficiency virus into the humans, resulting in current HIV pandemic (Worobey *et al.*, 2008).

In summary, many factors underlie the emergence of new human infections caused by viruses, including changes in human demographics and behavior, microbial adaptation and evolution, ecological and environmental changes. Clearly understanding of these determinants is needed for prevention and control of emerging viral diseases. New viral diseases will continue to emerge in humans in the future but when and where they occur is unknown. Survey of the circulating zoonotic viruses, assessment of the risk to the human and animal populations, with effective communications networks, developing new vaccines and antiviral agents, along with an increasing understanding

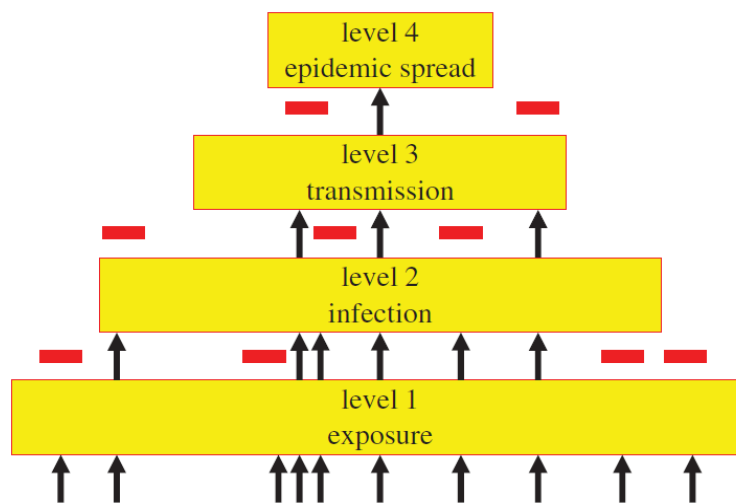
of viral population dynamics will contribute to either delaying viral emergences or minimizing their impact once they occur (Domingo 2010).

### 1.3. Pathogen pyramid

The concept of the pathogen pyramid (Figure 1.2) was suggested (Wolfe *et al.*, 2004) and developed (Wolfe *et al.*, 2007) to summarize disease emergence. The pyramid has four levels (Woolhouse & Gaunt, 2007) representing different degrees of human-pathogen interaction.

#### Level 1: Exposure

This stage represents the exposure of humans to a microbe but this does not result in a particular infection in humans. The source of emerging viruses is most likely to be other mammals or birds. The exposure can be via contact with materials containing the microbe (blood, saliva, faeces) or contaminated food and water or via a vector. The exposure rate depends on both the distribution and ecology of the animal host and human activities as reviewed above.



**Figure 1.2.** The pathogen pyramid, adapted from (Woolhouse & Gaunt, 2007). Each level represents a different degree of interaction between pathogens and humans. Some pathogens are able to progress from one level to the next (arrows); others are prevented from doing so by biological or ecological barriers (bars).

#### Level 2: Infection

At this stage, a microbe is capable of infecting humans and may cause disease. Only a subset of the animal microbes that humans expose to can cross the 'species barrier' - the natural mechanisms that involve both the molecular biology of the microbe (e.g. is it capable of replicating in human cells) and the physiology of the exposed human (especially immunocompetence) - to infect humans. As mentioned above, 1,407 pathogen species, more than half of which are from other species of mammal, have been known to reach this stage. Moreover, it is notable that almost 70% of 374 pathogen species in dogs and cats are zoonotic (Cleaveland *et al.*, 2001). This indicates the species barrier (at least between humans and other mammals) may not always be profound.

### **Level 3: Transmission**

A subset of microbes from level 2 that can infect humans are also capable of transmission between humans. Transmission can be direct (e.g. via sexual contact) or indirect (e.g. via contaminated food) or via an arthropod vector. Despite the lack of information for many pathogen species, a literature search (Taylor *et al.*, 2001) suggested that at least 500 species can transmit between humans. The barriers to human-to-human transmission mainly reflect the human host-pathogen interaction, with focus of the possibility of the virus to access tissues from which it can exit the host, such as the upper respiratory tract, lower gut, urogenital tract, skin or blood.

### **Level 4: Epidemic Spread**

Pathogens that can progress to this stage are characterised by their ability of sufficient transmission between humans to cause major epidemics and/or become endemic in human populations without the requirement of animal host. Only a few pathogens that spill over from animals have epidemic or pandemic potential but the global impact could be devastating. It was estimated that 100 to 150 pathogen species are capable of causing major outbreaks within human populations (Woolhouse & Gowtage-Sequeria, 2005).

Level 3 and 4 can be differentiated using the concept of the basic reproduction number,  $R_0$ , defined as the average number of secondary cases of infection produced when a

primary case is introduced into a large population of previously unexposed hosts (Anderson & May, 1992). On average, for level 3, a single primary case will produce less than one ( $R_0 < 1$ ) secondary case. In contrast, at level 4, on average, more than one secondary case are produced from a single primary case ( $R_0 > 1$ ), leading to at least initially an exponential increase in the number of cases and possibly ultimately a major epidemic.

The barriers between level 3 and level 4 are both biological (pathogen infectivity, host susceptibility, the infectiousness of the infected host, the length of the infection) and epidemiological (the rate and pattern of contacts between infectious and susceptible hosts).

While relatively little is known about the factors driving the transition from one level to the next, clearly the human-animal interface is of paramount importance in the process. The possibility of cross-species transmission dramatically increases with the interaction between humans and animals through bushmeat practice, the trading of animal foods, animal husbandry practices, wet markets, the domestication of animals (Pike *et al.*, 2010).

## **1.4. Picornaviruses**

### **1.4.1. General aspects**

Picornaviruses belong to the family *Picornaviridae*, one of five virus families of the order *Picornavirales* (which also includes *Dicistroviridae*, *Marnaviridae*, *Iflaviridae* and *Secoviridae*) (Sanfaçon *et al.*, 2011). Members of the order have a single stranded positive sense ribonucleic acid (RNA) genome and are grouped together on the basis of similar capsid structure and viral life cycles. The family is named for the fact that all members are extremely small (pico) RNA viruses.

Picornaviruses have a wide range of human and animal hosts (Table 1.1) with infections ranging from asymptomatic to severe illnesses such as gastroenteritis, aseptic meningitis, encephalitis, the common cold, hand-foot-and-mouth disease, conjunctivitis, herpangina, myositis, myocarditis, hepatitis, foot-and-mouth disease (Knowles *et al.*, 2010; Yin-Murphy & Almond, 1996).



**Table 1.1.** Members of the family *Picornaviridae* and their natural hosts

Genera	Species	Number of types <sup>a</sup>	Host
<i>Apthovirus</i>	<i>Foot and mouth disease virus (FMDV)</i>	7	Cloven-footed animals (cattle and pigs)
	<i>Bovine rhinitis A virus (BRAV)</i>	2	Cows
	<i>Bovine rhinitis B virus (BRBV)</i>	3	Cows
	<i>Equine rhinitis A virus (ERAV)</i>	1	Horses
<i>Aquamavirus</i>	<i>Aquamavirus A</i>	1	Seals
<i>Avihepatovirus</i>	<i>Avihepatovirus A</i>	3	Ducks
<i>Avisivirus</i>	<i>Avisivirus A</i>	1	Turkeys
<i>Cardiovirus</i>	<i>Cardiovirus A</i>	2	Pigs, aardvarks, domesticated wild boars, mice, tigers, primates, humans, elephant
	<i>Cardiovirus B</i>	15	Rodents, humans
	<i>Cardiovirus C</i>	2	Rats
<i>Cosavirus</i>	<i>Cosavirus A</i>	24	Humans
<i>Dicipivirus</i>	<i>Dicipivirus A</i>	1	Dogs
<i>Enterovirus</i>	<i>Enterovirus A (EV-A)</i>	25	Humans, primates
	<i>Enterovirus B (EV-B)</i>	63	Humans, primates, pigs
	<i>Enterovirus C (EV-C)</i>	23	Humans, primates
	<i>Enterovirus D (EV-D)</i>	5	Humans, primates
	<i>Enterovirus E (EV-E)</i>	4	Cows
	<i>Enterovirus F (EV-F)</i>	6	Cows, possums
	<i>Enterovirus G (EV-G)</i>	16	Pigs, sheep
	<i>Enterovirus H (EV-H)</i>	1	Primates
	<i>Enterovirus J (EV-J)</i>	6	Primates
	<i>Rhinovirus A</i>	80	Humans
	<i>Rhinovirus B</i>	32	Humans
	<i>Rhinovirus C</i>	55	Humans
<i>Erbovirus</i>	<i>Erbovirus A</i>	3	Horses
<i>Gallivirus</i>	<i>Gallivirus A</i>	1	Chickens, turkeys
<i>Hepatovirus</i>	<i>Hepatovirus A</i>	1	Humans, primates
<i>Hunnivirus</i>	<i>Hunnivirus A</i>	4	Cattle, sheep, rats
<i>Kobuvirus</i>	<i>Aichivirus A</i>	1	Humans
	<i>Aichivirus B</i>	2	Cows, sheep
	<i>Aichivirus C</i>	1	Pigs, wild boars
<i>Kunsagivirus</i>	<i>Kunsagivirus A</i>	1	Rollers
<i>Megrivirus</i>	<i>Melegrivirus A</i>	1	Turkeys
<i>Mischivirus</i>	<i>Mischivirus A</i>	1	Bats
<i>Mosavirus</i>	<i>Mosavirus A</i>	2	Mice, rollers
<i>Oscivirus</i>	<i>Oscivirus A</i>	2	Birds
<i>Parechovirus</i>	<i>Parechovirus A</i>	16	Humans, primates
	<i>Parechovirus B</i>	4	Voies
<i>Pasivirus</i>	<i>Pasivirus A</i>	3	Pigs
<i>Passerivirus</i>	<i>Passerivirus A</i>	1	Birds
<i>Rosavirus</i>	<i>Rosavirus A</i>	2	Humans, mice
<i>Sakobuvirus</i>	<i>Sakobuvirus A</i>	1	Cats
<i>Salivirus</i>	<i>Salivirus A</i>	2	Humans, primates
<i>Sapelovirus</i>	<i>Sapelovirus A</i>	1	Pigs
	<i>Sapelovirus B</i>	3	Primates
<i>Senecavirus</i>	<i>Senecavirus A</i>	1	Pigs
<i>Sicinivirus</i>	<i>Sicinivirus A</i>	1	Chickens
<i>Teschovirus</i>	<i>Teschovirus A</i>	13	Pigs, wild boars
<i>Tremovirus</i>	<i>Tremovirus A</i>	1	Chickens, turkeys, pheasants

<sup>a</sup> Information included on number of types and known host species is adapted from information available

on the Picornavirus Study Group website ([www.picornaviridae.com](http://www.picornaviridae.com), accessed November 10, 2015).

Transmission is horizontal through either faeco-oral or respiratory routes or both. Some picornaviruses can remain infectious for variable periods in the environment outside their host cells and, therefore, may be transmitted via contaminated surfaces (Knowles *et al.*, 2010).

#### **1.4.2. Classification**

*Picornaviridae* is one of the most diverse virus families, with 50 species grouped into 29 genera (Table 1.1) (<http://www.picornaviridae.com>, accessed November 10, 2015). The classification of RNA viruses has particular difficulties due to their frequent recombination and high mutation rates during replication of the genome. Picornavirus classification is still in flux because a number of candidates for novel viruses belonging to this family have recently been discovered and described (Knowles *et al.*, 2012). The discovery of novel picornaviruses that are unassigned and appear to be intermediate between known genera may result in the joining of some genera (as the case of entero- and rhinoviruses). Picornaviruses were formerly classified using properties, some of which such as pH stability, host specificity and receptor usage have been found unreliable for measuring relationships. The classification of picornaviruses is now based on phylogenetics, minor differences in genome organization and gene content (Knowles *et al.*, 2010).

The length of sequences (whole genomes or smaller regions) used for classification will depend on (i) the taxonomic level being studied and (ii) whether recombination with other picornaviruses has been reported. For genera and species levels, P1 (the precursor of proteins 1A – 1D) and the combined 2C and 3CD regions are used as one of the main criteria for classification as P1 has the antigenic properties, the traditional way of defining virus identity, while 2C + 3CD is usually the most conserved and readily aligned region of the genome (Knowles *et al.*, 2010). According to the Ninth Report of the International Committee on Taxonomy of Viruses (Knowles *et al.*, 2012), members of a picornavirus species must share i) a natural host range, ii) a common genome organization, iii) greater than 70% identity in the amino acid

sequences of both the polyprotein and the combined 2C + 3CD polypeptides. The P1 thresholds of species vary between genera.

Because distinct viral genotypes or even minor genetic variations can lead to large changes in viral pathogenicity (Blinkova *et al.*, 2009; Zoll *et al.*, 2009a), it is necessary to identify picornaviruses to type level which for many years, serological methods were the standard technique. Serotyping, which includes virus isolation and subsequent neutralization of cultured viruses, is time-consuming (isolation and serotype determination serotyping of EV takes 1 – 2 weeks). Moreover, virus isolation requiring multiple cell lines and highly optimised cell culture methods is labour intensive (Muir *et al.*, 1998). Some viruses (such as members of *Rhinovirus C*) are difficult or even impossible to isolate (Bochkov & Gern, 2012). The neutralization step requires standardised sera which are in limited supply.

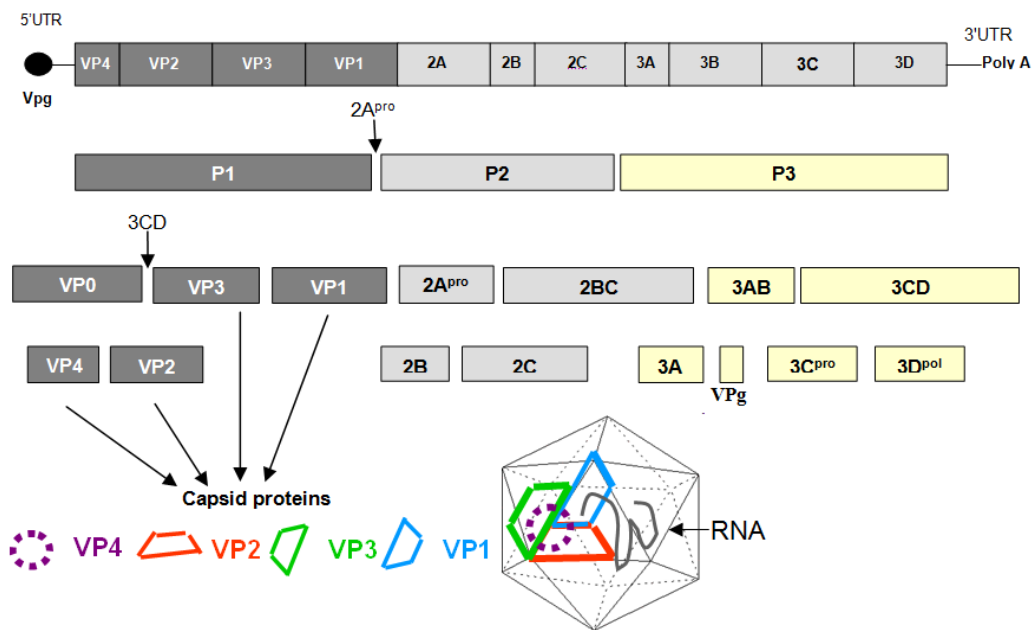
The ability of PCR-based methods to directly identify serotypes in clinical specimens overcomes these disadvantages of cell culture-based methods for serotyping (Leitch *et al.*, 2009). The EV VP1 gene encoding for a protein that contains a number of neutralization domains has been proved to correspond with results obtained by neutralization (serotype) test, and hence, with phylogenetic lineage (Kiang *et al.*, 2009; Kilpatrick *et al.*, 1998; Oberste *et al.*, 1999). In such manner, both genotypic identification and assignment of types are based on the sequences of VP1 (Stanway *et al.*, 2005). Practical thresholds for EV type identification include VP1 identity of at least 75% in the nucleotide sequence or 88% in amino acid sequence (Oberste *et al.*, 1999).

#### **1.4.3. Genome organisation and proteolytic processing**

The genomes of all picornaviruses consist of single-stranded, positive sense, nonsegmented RNA. The known genomes range in length from 7,032 bases (*Avian encephalomyelitis* virus) to 8,828 bases (*Erbovirus*) and share a highly conserved organization which is also a defining characteristic of the family. The genomes comprise of 5' and 3' untranslated terminal regions flanking a single open reading frame (Palmenberg *et al.*, 2010). The 3' poly(A) tail is genetically encoded and essential for genome replication (Herold & Andino, 2001). The 5' end of the RNA

genome covalently links to a small viral genome-linked protein (VPg), which acts as a primer for genome replication. The VPg is encoded by the short and well conserved 3B gene. Unlike other picornaviruses, FMDV and aquamavirus A genomes consist of more than one sequential paralogous VPg genes (Forss & Schaller, 1982; Knowles & Wadsworth, 2010). In contrast to eukaryotic messenger RNA, picornavirus RNA lacks a methylated 5' cap structure. The cap-independent translation occurs via ribosome binding to an internal ribosome entry site (IRES) located in the 5'UTR of the viral RNA (Palmenberg *et al.*, 2010).

The single open reading frame encodes for a co-translationally cleaved polyprotein. Therefore, full-length translation products are not present in the infected cells (Martinez-Salas & Ryan, 2010). The translated polyprotein is cleaved into three regions known as P1, P2 and P3 (Rueckert & Wimmer, 1984). These regions are then processed by viral proteases and autocatalytic cleavages (Figure 1.3) to produce capsid components and non-structural proteins.

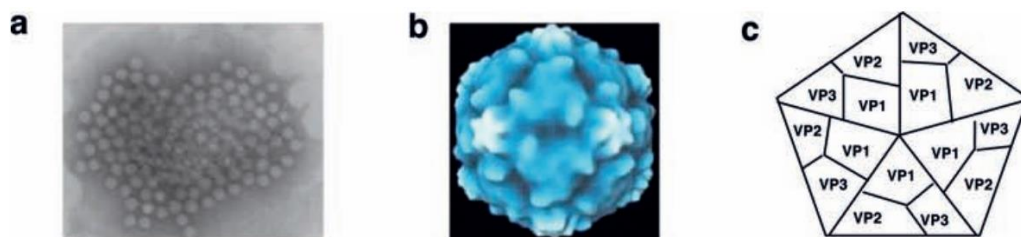


**Figure 1.3.** Genome organisation, polyprotein processing cascade and capsid architecture of EV. Several notable differences between proteolytic processing events in other picornavirus genera exist. Adapted from (Hober *et al.*, 2013). Organisation of picornavirus genome is shown on the top line. The translation of the genome results in a polyprotein which is cleaved into four capsid proteins (dark gray) and seven non-structural proteins (light gray and yellow).

As ribosomes traverse the middle region of the genome, the junction between the P1 and P2 coding regions is cleaved by the 2A proteinase in *Enterovirus* and by the 3C proteinase in *Cardiovirus*, *Parechovirus*, *Hepatovirus* and *Aphovirus*, whose 2A protein does not have proteolytic activity. In some picornavirus genera (*Aphovirus*, *Cardiovirus*, *Erbovirus*, *Kobuvirus* and *Sapelovirus*), the P1 region is preceded by a leader protein (L). However, only the leader protein of *Aphovirus* and *Erbovirus* functions as a proteinase (Martinez-Salas & Ryan, 2010). Next, 3C proteinase catalyzes the cleavage between the P2 from P3 regions in all picornaviruses. Subsequent cleavage events are processed either by the 3C proteinase or its precursor (3CDpro), that also possesses proteolytic activity. The final event is the maturation of VP0 to form VP4 and VP2 during the last stages of virion morphogenesis. VP0 cleavage is thought to be autocatalytic (Fry & Stuart, 2010), as the VP0 scissile bond is located deep within a maturing particle and is inaccessible to exogenous proteases (Arnold *et al.*, 1987).

#### 1.4.4. Virion structure

As the name indicates, picornaviruses are small viruses (28 – 30nm in diameter) with positive-stranded RNA genome surrounded by a protein shell (capsid). Their infectivity is not affected by inorganic solvents as they lack a lipid envelope. Despite the genetic diversity of the capsid coding sequences, picornaviruses share the conserved capsid structure across the family (Racaniello, 2007). The three dimensional structure of the capsid has been determined for some picornaviruses of different genera such as EVs (Filman *et al.*, 1998; Garriga *et al.*, 2012; Hadfield *et al.*, 1997; Medappa *et al.*, 1971; Oliveira *et al.*, 1993), senecavirus (Venkataraman *et al.*, 2008), FMDV (Acharya *et al.*, 1989; Lea *et al.*, 1995; Lea *et al.*, 1994) and cardioviruses (Grant *et al.*, 1992; Luo *et al.*, 1992).



**Figure 1.4.** The structure of human rhinovirus virions. Adapted from (Dreschers *et al.*, 2007). (a): Electron microscopy analysis of purified rhinoviruses. (b): Structural model of rhinovirus obtained from cryoelectron microscopic data. (c): Positions of capsid proteins VP1, VP2 and VP3 on the rhinovirus capsid.

The capsid of most picornaviruses is composed of 60 copies of the four proteins VP1 to VP4. For parechoviruses and kobuviruses, VP0 remains uncleaved and their virions contain 60 copies of only three proteins, VP0, VP1 and VP3 (Stanway *et al.*, 1994). The external capsid proteins, VP1 to VP3 make up an 8 stranded antiparallel beta barrel (jelly roll) widely found in RNA viruses. The VP4 protein is attached to the inner surface of the capsid (Fry & Stuart, 2010).

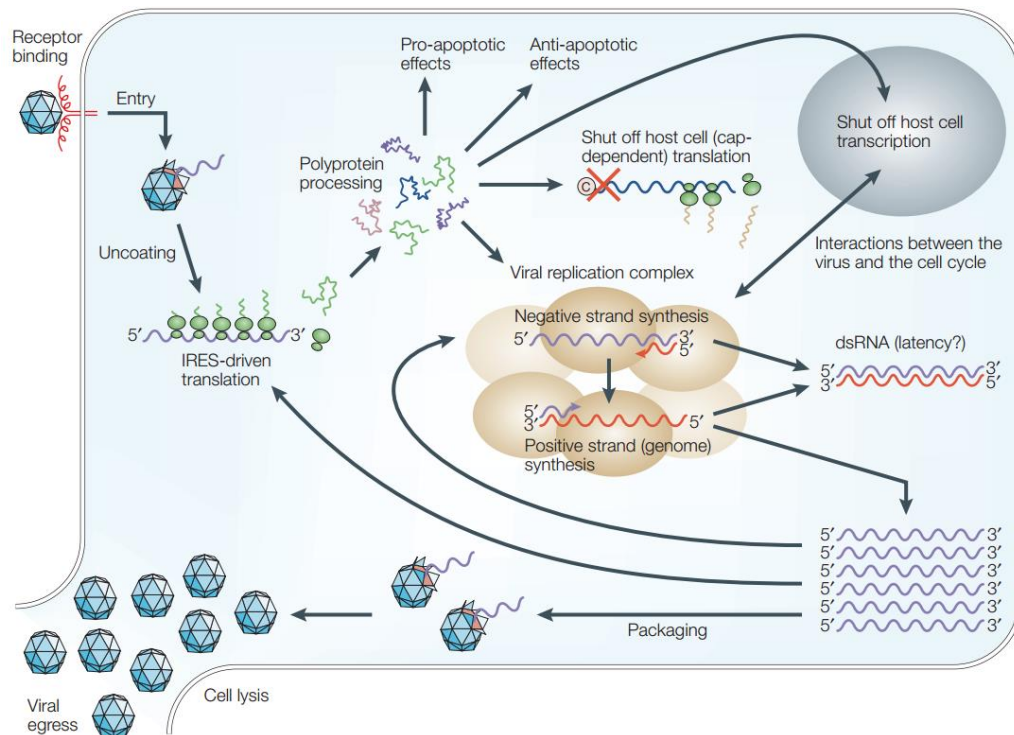
The capsid proteins are assembled in a stepwise process. First, VP0, VP1 and VP3 form a protomer. After that, a pentamer is produced from five protomers. Twelve pentamers then assemble to form a rigid pseudo-spherical structure with icosahedral symmetry surrounding a newly synthesized RNA (Hellen & Wimmer, 1995). The maturation cleavage of VP0 into VP4 and VP2 confers stability on the particle. The capsid is stable to protect the fragile RNA genome but, at the appropriate signal, is also flexible (Smyth & Martin, 2002) to dissociate or undergo conformational changes described as “breathing” during which VP4 and the N-terminus of VP1 become exposed (Lewis *et al.*, 1998; Li *et al.*, 1994). These changes are necessary for viral RNA genome release.

The virion has five fold, three fold and two fold axes of symmetry. In most picornaviruses, a circular depression or “canyon” formed by the beta barrels of the VP1 protein around each icosahedral fivefold axis of symmetry is a receptor-binding site (Colonno *et al.*, 1988; Hewat *et al.*, 2000). Aphoviruses lack the canyon and bind to receptors via flexible surface loops (Acharya *et al.*, 1989). The VP1  $\beta$ -barrel of many picornaviruses also contains a hydrophobic pocket which is occupied by “pocket factors” (Oliveira *et al.*, 1993; Verdaguer *et al.*, 2000). Antiviral drugs such as hydrophobic antiviral WIN compounds, which bind tightly to this pocket, inhibit the uncoating and subsequent delivery of the RNA genome into the host cell (Smith *et al.*, 1986).

#### **1.4.5. Life cycles of picornaviruses**

The replication of picornaviruses takes place in the cytoplasm of the host cell. Binding of the virus to specific host cell receptors triggers irreversible conformational alterations in the capsid which results in the cell entry and release of the viral RNA

genome into the cytoplasm. The genome is first translated to produce the viral proteins needed for genome replication. After negative strands are synthesized from the RNA genome, they are used as templates for positive strand RNA synthesis. Capsid proteins and newly synthesized viral RNA genomes assemble into mature virions which are then released from the cell. The entire cycle (Figure 1.5) completes in around 5 – 10 hours (Racaniello, 2007).



**Figure 1.5.** Summary of the picornavirus life cycle. Adapted from (Whitton *et al.*, 2005). The figure summarises key events in the replication of picornaviruses and the main events are described in the text below. © represents the cap present on most cellular mRNA.

Picornaviruses can use a wide variety of cell surface receptors for binding and cell entry. The receptor usage profile of picornaviruses is complex and receptor specificities of some major picornavirus species are summarised in Table 1.2. Cell surface receptor specificity is an essential factor determining cell/tissue tropism and, thereby, defining host species and pathogenesis. However, receptor expression seems not to be the sole determinant of tissue tropism because cellular receptors used by picornaviruses are expressed in many tissues but viral tropism may be limited to just some of those tissues. For example, both ICAM-1 (exploited by HRV) and CD155 (PV receptor) are expressed in many tissue types throughout the body but HRV and PV primarily replicate in the respiratory and gastrointestinal tracts, respectively.

A picornavirus may use multiple routes of entry, depending on the target cell and the receptors available on that cell (Bergelson & Coyne, 2013). Picornaviruses have no lipid membrane that can directly fuse with the host cell plasma membrane for cell entry. Instead, picornaviruses utilize a variety of mechanisms for effective deliver of their genome into the cytoplasm. Picornaviruses enter the cell by different mechanisms of endocytosis and subsequent release from vesicles (Brandenburg *et al.*, 2007; Tuthill *et al.*, 2010). For aphthoviruses and some HRV, the endocytosis is clathrin-mediated (Tuthill *et al.*, 2010). Some EVs undergo endocytotic uptake via a caveolin mediated pathway, while PVs rely on a caveolin, clathrin-independent process (Brandenburg *et al.*, 2007; DeTulleo & Kirchhausen, 1998).

**Table 1.2.** Receptors used by selected picornaviruses

Receptor	Virus	Natural host
CD155	Poliovirus	Humans
ICAM-1	Rhinovirus (major group)	Humans
LDLR	Rhinovirus (minor group)	Humans
Coxsackie and adenovirus receptor (CAR)	Coxsackie viruses	Humans
DAF/CD55	Coxsackie viruses, EV70	Humans
Murine vascular cell adhesion molecular 1 (VCAM-1)	Encephalomyocarditis virus	Rodents
Integrins	FMDV Parechovirus	Cloven footed animals Humans
Sialic acid	Cardioviruses ERAV EV70	Rodents Horses Humans
Human P-selectin glycoprotein ligand 1	EV71	Humans
HAV-cr1	Hepatitis A virus	Humans

Information adapted from (Bergelson, 2010; Pallansch & Roos, 2007; Racaniello, 2007; Tuthill *et al.*, 2010).

The uncoating of the RNA genome is regulated by cues from receptors (as for PVs and major group HRVs) and/or chemical cues such as low pH as in the case of minor group HRVs (Suomalainen & Greber, 2013). Binding of PVs and the major group HRVs to their receptors (CD155 and ICAM-1, respectively) induces externalization of the N-terminus of VP1 and release of the N-myristoylated VP4 from the virion (or capsid breathing) before or during virus uptake by endocytosis (Brandenburg *et al.*, 2007; DeTulleo & Kirchhausen, 1998). In contrast, minor HRV such as HRV-2 do not receive sufficient uncoating cues from binding to their receptor (LDLR) and must also depend on acidification (pH 5.4) of the late endosomes to undergo antigenic



conversion (Brabec *et al.*, 2003; Garriga *et al.*, 2012; Schober *et al.*, 1998). The exposed VP1 tethers the virus to the endosomal membrane, and VP4 forms an aqueous pore in the membrane which is thought to be the entry of viral RNA into the cytoplasm (Danthi *et al.*, 2003; Davis *et al.*, 2008b; Fricks & Hogle, 1990). Capsid breathing can be blocked by uncoating inhibitors, such as pleconaril or WIN compounds. These bind to the receptor-docking cavity in the capsid, stabilize the capsid conformation, thereby block the release of VP4 and RNA from the virus (Lewis *et al.*, 1998).

Once in the cytoplasm, the viral RNA must be first translated in order to produce the viral proteins necessary for genome replication. These proteins are not naturally available in the host cell. When sufficient amounts of viral proteins have been produced, the switch from translation to replication occurs as viral genomes that are being actively translated cannot serve as templates for RNA synthesis (Barton *et al.*, 1999; Gamarnik & Andino, 1998). The host protein poly(rC) binding protein and the uncleaved viral protease polymerase precursor 3CD bind to the 5' cloverleaf structure of the RNA genome to form a ribonucleoprotein (RNP) complex (Andino *et al.*, 1990; Rohll *et al.*, 1994). This complex interacts with the poly(A) binding protein that is bound to the 3' end of the viral genome. As a result of this interaction, the ends of the viral RNA link together to form a circular RNP complex, which in turn initiates negative strand RNA synthesis from the 3' polyA region (Herold & Andino, 2001). The viral RNA dependent RNA polymerase, encoded by the 3D gene, is responsible for RNA synthesis. The negative strand then functions as a template for synthesis of positive strand, possibly after being anchored in the membranous replication complex by viral protein 2C (Banerjee *et al.*, 1997). The viral polymerase uses the uridylated protein VPg (VPg-pU-pU) as a primer for RNA synthesis which takes place within replication complexes bound to smooth membrane vesicles (Bienz *et al.*, 1994). The compartmentalization of RNA replication may enhance replication efficiency by localisation of all required components and protection from host defences (e.g. a dsRNA activated immune response) during the replication cycle (Racaniello, 2007).

Some of the newly synthesized positive stranded RNA viral genomes are translated to produce viral capsid components. Each positive-sense RNA genome is linked to a VPg

protein and enclosed in a capsid. The final event prior to cell lysis is the cleavage of VP0 to yield the mature capsid proteins VP4 and VP2 (Lee *et al.*, 1993).

#### **1.4.6. Recombination in the evolution of picornaviruses**

As mentioned, recombination is the process of genetic exchange between different organisms to create a chimeric genome. Recombination can be inferred indirectly from the identification of separate evolutionary origins of different genome regions created by a recombination event. A limitation of these observational methods is that they are unable to detect recombination between identical or very closely related parents, or in case the recombination parents no longer exist (Simmonds, 2010). Non-viable recombinants that are generated randomly within a single replication cycle cannot usually be detected either. Recombination in picornaviruses can be classified as homologous (between homologous parental RNAs, crossovers at homologous sites), aberrant homologous (between similar viruses without maintaining strict alignment) or nonhomologous (between unrelated RNA sequences) (Lai, 1992). Due to the likelihood of generating viable recombinants, homologous recombination is most frequently observed (Holmblat *et al.*, 2014).

The most widely accepted recombination model in RNA viruses is the copy-choice model. In this process, the RNA dependent RNA polymerase switches from one RNA molecule to another during synthesis while still binding to the nascent RNA. Consequently, an RNA molecule with mixed ancestry is generated (Simon-Loriere & Holmes, 2011). This mechanism requires coinfection of the same host cell by two different viruses whose replication takes place simultaneously within the same cellular compartment. The nascent strand dissociation may be promoted by RNA secondary structure or nucleotide misincorporations which causes the elongation pausing (Agol, 1997). Another proposed mechanism of recombination which is replication independent is the breakage and rejoining of RNA strands (Gmyl *et al.*, 2003).

Natural recombination is very frequent in picornaviruses and has been well documented in different picornavirus genera such as *Aphthovirus* (Heath *et al.*, 2006; Simmonds, 2006), *Parechovirus* (Benschop *et al.*, 2008c; Calvert *et al.*, 2010; Chen *et al.*, 2015; Zoll *et al.*, 2009b), *Teschovirus* (Simmonds, 2006; Wang *et al.*, 2010),

*Cardiovirus* (Naeem *et al.*, 2014; Ren *et al.*, 2013), *Kobuvirus* (Fan *et al.*, 2013) and *Enterovirus* (Gaunt *et al.*, 2015; Liu *et al.*, 2014; Lukashev *et al.*, 2003; McIntyre *et al.*, 2013a; Oberste *et al.*, 2004a; Oprisan *et al.*, 2002; Santti *et al.*, 1999; Simmonds & Welch, 2006; Smura *et al.*, 2014; Tang *et al.*, 2014; van der Sanden *et al.*, 2011). Documented recombination events in these genera tended to occur in the 5'UTR and the non-structural coding regions.

There is evidence that picornaviruses benefit from recombination. A recent study suggested recombination may play a role in the emergence and pathogenicity of CVA6 associated with several outbreaks worldwide of atypical HFMD or eczema herpeticum in the UK (Gaunt *et al.*, 2015). More convincingly, natural recombination between oral live-attenuated poliovirus vaccine strains and circulating EV generated pathogenic vaccine-derived PVs that subsequently caused outbreaks of vaccine associated poliomyelitis, implicating difficulties for the PV eradication campaign (Cuervo *et al.*, 2001; Guillot *et al.*, 2000; Jegouic *et al.*, 2009; Simmonds & Welch, 2006). Understanding the frequency and nature of naturally occurring recombination events in the circulation of a virus is, therefore, important for development and implementation of therapeutic strategies and potential vaccines.

## **1.5. Picornavirus genera in this study**

### **1.5.1. *Enterovirus***

EVs are among the most common viruses infecting humans and are associated with a broad spectrum of clinical syndromes, ranging from minor febrile illness to severe and potentially fatal diseases (Bessaud *et al.*, 2008). The EVs originally included only human viruses and were classified into four groups on the basis of their pathogenesis in humans and experimental animals: polioviruses (PV), coxsackieviruses A (CVA), coxsackieviruses B (CVB), and echoviruses. PV can infect primates although they are unlikely reservoirs in nature (Dowdle & Birmingham, 1997). CVA were discovered from stool samples of children with paralysis in the town of Cocksackie, New York (Dalldorf & Sickles, 1948). CVB were isolated from cases of aseptic meningitis (Melnick *et al.*, 1949). CVA caused a flaccid paralysis while infection with CVB resulted in a spastic paralysis and severe systemic infection in suckling mice. The last

classical group of human EV are named echoviruses (enteric cytopathogenic human orphan viruses) due to their initial lack of association with clinical disease in either humans or laboratory animals. Echoviruses were first isolated from tissue culture samples (Robbins *et al.*, 1951).

As more sequence data were available, it was realized that these subgroups did not correlate with observed phylogenetic relationships (Hyypiä *et al.*, 1997). In addition, each type may cause a wide spectrum of symptoms, making classification using clinical terms impossible. Therefore, human EVs were reclassified into four species (HEV-A – D) on the basis of sequence identity and phylogenetic relationships. Within each species, individual types were identified either by neutralization test or sequence comparison of the VP1 region. This new classification method has led to the recognition that many animal viruses also belong to the *Enterovirus* genus (Table 1.1). Indeed, many EVs infecting non-human primates are members of human EV species HEV-A – D (Harvala *et al.*, 2012; Harvala *et al.*, 2011b; Sadeuh-Mba *et al.*, 2014). This has resulted in the removal of the host species from the enterovirus names.

The *Enterovirus* genus consists of 12 species, including 3 rhinovirus species (A to C) and 9 enterovirus species (A to J) that infect humans, pigs, cows and nonhuman primates ([www.picornaviridae.com](http://www.picornaviridae.com), accessed November 10, 2015 (Knowles *et al.*, 2010)). The genus is notable for its high genetic and antigenic diversity with more than 300 serotypes (Table 1.1). Among these, some human coxsackieviruses have been isolated from the blood of a rabbit (CVA4) (O'Connor & Morris, 1955), the brain of a fox (CVA5) (Makower & Skurska, 1957), the intestine of a bat (CVB3) and rats (CVB3 & CVB6) (Gregorio *et al.*, 1972), pigs (CVB4, CVA20) (Grew *et al.*, 1970; Lomakina *et al.*, 2015), dogs (CVB3, CVB5, CVB6) and chickens (CVB4) (Graves & Oppenheimer, 1975). Since the discovery of simian enteroviruses in the 1950s from primate cell cultures, many more enterovirus serotypes have been identified, mostly from chimpanzees, gorillas and the Old World monkeys in Bangladesh and Cameroon (Harvala *et al.*, 2011b; Harvala *et al.*, 2014; Oberste *et al.*, 2013a; Oberste *et al.*, 2013b; Sadeuh-Mba *et al.*, 2014). They are normally genetically separate from those infecting humans but a number of serotypes in enterovirus species A (CVA2, CVA5, EV71, EV76, EV89, EV90, EV119), B (CVB1, CVB3, CVB5, CVB6, E11, E15, E19, E20,

E21, E24, E29, E33, EV74, EV75, EV97, B82), C (CVA13, CVA19, CVA20, CVA22, CVA24, Sabin-3, EV99) and D (D111) have been detected in both human and non-human primate samples. In addition, Harvala *et al.* (2012) showed high seroprevalence of human serotypes E11, EV76, EV94 in apes and Old World monkeys. These evidences of cross-species transmission of EVs between primates have raised concerns about the primate reservoirs as a potential source of emerging EV infections. However, the transfer direction and the potential primate sources of zoonotic EV infections in humans require further investigation (Harvala *et al.*, 2011b).

### **1.5.2. Parechovirus**

The genus *Parechovirus* is comprised of two species, *Parechovirus A* (formerly named *Human parechovirus* - HPeV) and *Parechovirus B* (formerly named *Ljungan virus*) and a candidate species, Sebokele virus 1 from rodents ([www.picornaviridae.com](http://www.picornaviridae.com), accessed November 10, 2015). A fourth parechovirus from ferrets has recently been described (Smits *et al.*, 2013).

*Ljungan virus* (LV) was first isolated from bank voles at the Ljungan River in central Sweden in efforts to find the causative agent of lethal myocarditis in six Swedish orienteers. Antibodies against one of the three LV isolates were detected in four of five sera from the orienteer patients (Niklasson *et al.*, 1999). Another seroprevalence study showed evidence of LV specific antibodies in 14/37 (38%) patient serum samples (Jääskeläinen *et al.*, 2013b). However, no LV RNA has been detected in human samples. LV is common (24.4% positive by PCR) in rodents in the UK (Salisbury *et al.*, 2014) and was also detected in wild voles or mice in the USA (Johansson *et al.*, 2003) and Italy (Hauffe *et al.*, 2010). LV is associated with diseases such as myocarditis, encephalitis, pregnancy related diseases, and diabetes in several species of wild rodents (Niklasson *et al.*, 2006).

The respiratory and gastrointestinal tract is thought to be the primary site of HPeV replication. Replication in the intestine leads to prolonged shedding of the virus in faeces which can be detected by virus isolation and polymerase chain reaction (PCR) methods (Harvala & Simmonds, 2009). Sixteen types (HPeV-1 – 16) of *Parechovirus A* have been described on the basis of the phylogenetic analyses of the VP1 encoding

region (Esposito *et al.*, 2014). HPeV-1 and HPeV-2 were formerly classified in the *Enterovirus* genus as echovirus 22 and 23, respectively. They were then reclassified as members of a new genus due to differences from the *Enterovirus* genus (King *et al.*, 1999).

HPeVs have recently been recognized as important viral pathogens causing various illnesses (Walters, 2011). The majority of published reports relate to types HPeV-1 to -8 from children (Benschop *et al.*, 2008b; Ghazi *et al.*, 2012; Harvala *et al.*, 2011a). In pediatrics, HPeVs are associated with a variety of symptoms from mild gastrointestinal or respiratory illness (by HPeV-1 – 3) to more serious diseases such as myocarditis, encephalitis, pneumonia, meningitis, flaccid paralysis, Reye syndrome and fatal neonatal infection (HPeV-1 – 2) (Khetsuriani *et al.*, 2006; Stanway *et al.*, 2000). In infants, HPeV-3 is a significant cause of sepsis-like illness (as is HPeV-4) (Benschop *et al.*, 2006; Harvala *et al.*, 2011a; Jääskeläinen *et al.*, 2013a; Sharp *et al.*, 2013) and encephalitis (Verboon-Maciolek *et al.*, 2008). HPeV-5 – 8 seem to cause diseases similar to those by HPeV1 and HPeV2 (Levorson & Jantusch, 2009).

Seroprevalence studies show HPeV infections are prevalent in the general population. Specific antibodies against HPeV serotypes 1, 2, 4 – 6 have been detected at high prevalence (35 – 99%) in children of 10 years old or younger (Korpela & Hyypiä, 1998; Westerhuis *et al.*, 2013). HPeV1-specific antibodies were also reported in 97 – 99% of the adult population (Korpela & Hyypiä, 1998; Tauriainen *et al.*, 2007). The extremely high seroprevalence of HPeV-1 and -2 in young children indicates that severe disease associations with infection are rare (Harvala & Simmonds, 2009), as confirmed by a recent longitudinal study of a group of 102 infants in Norway. HPeVs (mainly type 1) were detected in 11% of 1941 stool samples without significant association with coughing, sneezing, fever, diarrhoea or vomiting (Tapia *et al.*, 2008).

HPeV-1 and -6 were recently detected in faeces of 6/116 diarrhoeic monkeys on a monkey farm in China (Shan *et al.*, 2010). Another study (Oberste *et al.*, 2013b) detected HPeV in 21/618 faecal samples from rhesus macaques with types identified as HPeV1, HPeV4, HPeV5, HPeV12, HPeV14 and HPeV15. These findings indicate

monkeys may serve as reservoirs for zoonotic transmissions of HPeVs and as models for studies of HPeV pathogenesis.

Although HPeVs are frequently isolated from respiratory samples and are thought to be associated with disease, HPeVs are not yet commonly a part of respiratory screening (Esposito *et al.*, 2014; Harvala *et al.*, 2008). This may have resulted in very limited information on the infection and molecular characteristics of HPeVs in Vietnam.

### 1.5.3. *Cosavirus*

*Cosavirus* (common stool-associated virus) is a new genus, which is currently composed of the species *Cosavirus A* (with 25 types) and four candidate species (*Cosavirus B* to *E*) ([www.picornaviridae.com](http://www.picornaviridae.com)). This genus has not only apparently wide geographic distribution with prevalence varying according to age and condition of the studied populations, and geographic area, but also a wide genetic diversity. Cosaviruses were originally identified in 2008 in the faeces of children with non-polio acute flaccid paralysis (AFP) and healthy children in South Asia (Kapoor *et al.*, 2008). Based on the genetic comparisons, the authors proposed that the detected cosavirus variants comprise 4 different species (*Cosavirus A – D*). Similarly, a novel cosavirus detected in Australia was proposed to represent a new species (*Cosavirus E*) (Holtz *et al.*, 2008).

The wide genetic diversity of human cosaviruses (HCoSVs) complicates studies on their disease association since different serotypes or even minor genetic variations can result in large changes in viral pathogenicity (as shown by the extensively studied EVs) (Kapusinszky *et al.*, 2012). Although studies show that cosaviruses are widely distributed across different regions of the world, their clinical significance is uncertain. In support for potential disease associations of the virus, a study in Tunisia showed significantly higher prevalence of HCoSVs in AFP patients (42.8%) than that in healthy controls (25.4%) (Rezig *et al.*, 2015). HCoSV induced rapid cytopathic effects (CPE) in human embryonic lung MRC5 cell line with a remarkable decrease in live cells observed on the third day of CPE (Rezig *et al.*, 2014). A cosavirus infection was also reported in an Australian child (Holtz *et al.*, 2008), a Thai adult, both with diarrhoea (Khamrin *et al.*, 2012), and an Italian patient with chronic diarrhoea

associated with cosavirus persistent infection (Campanini *et al.*, 2013). However, HCoVSVs were detected with no statistically significant difference in prevalence between healthy controls and paralyzed children in Pakistan (Kapoor *et al.*, 2008) or children with diarrhoea in China (Dai *et al.*, 2010). In addition, a study in Brazil even showed higher prevalence of cosavirus in controls than in patients, frequent co-infections with established pathogens in diarrheal children, and low viral loads. These findings provide evidence against pathogenicity of HCoVSVs (Stöcker *et al.*, 2012).

#### **1.5.4. *Cardiovirus***

The *Cardiovirus* genus is made up of three species, *Cardiovirus A – C* (formerly named *Encephalomyocarditis virus*, *Theilovirus* and Boone cardiovirus, respectively). *Cardiovirus A* consists of two serotypes, named encephalomyocarditis virus 1 (EMCV-1) and the newly discovered EMCV-2. *Cardiovirus B* is comprised of 14 genotypes, which may also be different serotypes, including Theiler's murine encephalomyelitis virus (TMEV), Vilyuisk human encephalomyelitis virus (VHEV), Thera virus (TRV) and Saffold virus (SAFV) 1 to 11 (<http://www.picornaviridae.com>, accessed November 10, 2015).

EMCV-1 has been reported from a wide range of hosts including pigs (Joo, 1999), rodents (Wells & Gutter, 1989), cattle (Spradbrow & Chung, 1970), elephants (Grobler *et al.*, 1995), squirrels (Blanchard, 1987), baboons (Hubbard *et al.*, 1992), rhesus macques (Masek-Hammerman, 2012), chimpanzees (Jones *et al.*, 2011; Reddacliff *et al.*, 1997), lemurs (Canelli *et al.*, 2010) and humans (Kirkland *et al.*, 1989; Oberste *et al.*, 2009; Verlinde & Van Tangeren, 1953). Among these, rodents are thought to be the natural host of EMCV-1 (Tesh & Wallace, 1978) but pigs are the most commonly and severely infected domestic animals (Petrucelli *et al.*, 1991). EMCV can cause acute myocarditis in young piglets (Gainer, 1967) and reproductive failure in sows (Koenen *et al.*, 1991; Love & Grewal, 1986).

In contrast to the ability of EMCV-1 to infect a variety of hosts, *Cardiovirus B* shows a narrow host range (Philipps *et al.*, 2012). TMEVs were originally isolated from mice (Theiler, 1934) and rats (Hemelt *et al.*, 1974). TMEVs mostly cause asymptomatic infections of the digestive tract in mice, and rarely spread to the mouse central nervous



system (CNS). TMEV isolates are divided into two subgroups on the basis of their pathogenesis (Liang *et al.*, 2008). TMEVs with high neurovirulence (such as GDVII) cause a rapidly fatal encephalitis in mice, while the low-neurovirulence strains (e.g. BeAn, DA) cause persistent infection in the central nervous system of mice (Lehrich *et al.*, 1976; Lipton, 1975).

Whether cardioviruses can actually infect humans was unclear for a long time (Zoll *et al.*, 2009a). The first identified human cardiovirus, VHEV, was previously thought to cause degenerative neurological disease in inhabitants in Vilyuisk, Siberia, in the 1950s. The virus was isolated from mice inoculated with human clinical samples but has not been identified in any other patient with this disease since then. Using complement fixation test, Casals showed that mouse serum against VHEV cross-reacted with TMEV and weakly with EMCV (Casals, 1963). Its sequence showed closest relationship to TMEV, which causes a persistent infection of the central nervous system in mice. VHEV therefore may be a contaminating cardiovirus that originated from the mice used for isolation of the virus (Lipton, 2008). Similarly, detection of human EMCV is questionable (Oberste *et al.*, 2009).

Recently, however, there has been some strong evidence for the existence of human cardioviruses (Zoll *et al.*, 2009a). In 2007, a novel cardiovirus namely Saffold virus (SAFV) was discovered in a stool sample from a patient with fever of unknown origin (Jones *et al.*, 2007). The virus was subsequently detected by molecular methods in children with respiratory symptoms (Abed & Boivin, 2008; Tsukagoshi *et al.*, 2011) or gastroenteritis (Chiu *et al.*, 2010; Drexler *et al.*, 2008a; Ren *et al.*, 2009) and in both patients with nonpolio AFP and healthy children in Pakistan at high frequencies of 9 and 12%, respectively (Blinkova *et al.*, 2009). SAFVs identified so far are significantly divergent from all known animal cardioviruses, especially in the capsid coding region (Zoll *et al.*, 2009a).

Cell culture researches showed efficient growth of SAFV-3 in several cell lines with a clear cytopathic effect. This feature enabled a large-scale serological survey which revealed a high seroprevalence of 75% of SAFV-3 in children at 24 months and even higher (> 90%) in older children and adults (Zoll *et al.*, 2009a). More notably, SAFV-

3 was recently isolated from the cerebrospinal fluid (CSF) of a 9-year old boy with aseptic meningitis (Himeda *et al.*, 2011), while SAFV-2 was detected in the CSF, blood and myocardium of a child who died suddenly with no history of illness (Nielsen *et al.*, 2012). These findings indicate that SAFVs may have CNS tropism and disease association. SAFVs probably behave similarly to EVs, which cause mainly asymptomatic infections or nonspecific symptoms in other patients and rarely result in severe disease (Nielsen *et al.*, 2012). However, specific disease associations of the new human cardioviruses remain to be determined.

#### **1.5.5. Kobuvirus**

*Aichivirus A* (formerly *Aichi virus*), *Aichivirus B* (formerly *Bovine kobuvirus*) and *Aichivirus C* (porcine kobuvirus) are the three species of the genus *Kobuvirus*. Other kobuviruses from goat (Oem *et al.*, 2014) and European roller (Pankovics *et al.*, 2015) may represent two further candidate species. Kobu-like viruses have also been identified in bats (Li *et al.*, 2010).

*Aichivirus A* currently consists of one type, *Aichivirus 1* (AiV-1). Canine kobuvirus 1 (Carmona-Vicente *et al.*, 2013; Li *et al.*, 2011), feline kobuvirus 1 (Cho *et al.*, 2014; Cho *et al.*, 2015; Chung *et al.*, 2013) and murine kobuvirus 1 (Phan *et al.*, 2011) are most closely related to AiV-1 and probably represent novel types of this species (<http://www.picornaviridae.com>, accessed November 10, 2015).

Only AiV-1 is known to be able to infect humans. AiV-1 was first isolated from a stool specimen of a patient with oyster-associated nonbacterial gastroenteritis in 1989 in the Aichi District of Japan (Yamashita *et al.*, 1991). Surveys in Japan showed seroprevalence for AiV-1 increased with age, from 7.2% for persons aged 7 months to 4 years to about 80% in persons 35 years old (Yamashita *et al.*, 1993). By reverse transcription-PCR, a high detection frequency (20.1%) of aichivirus was reported from a study of 268 faecal specimens from patients in 12 of 37 gastroenteritis outbreaks in Japan, nearly all were associated with oysters (Yamashita *et al.*, 2000). AiV-1 RNA was detected at low frequency (0.5 – 3.5%) in samples mostly from patients with gastroenteritis in Pakistan (Yamashita *et al.*, 1995) and France (Ambert-Balay *et al.*, 2008), Japan, Bangladesh, Thailand and Vietnam (Pham *et al.*, 2007), Tunisia (Sdiri-

Loulizi *et al.*, 2008), Hungary (Reuter *et al.*, 2009), China (Yang *et al.*, 2009), Finland (Kaikkonen *et al.*, 2010) and Korea (Han *et al.*, 2014). Although no direct evidence for the pathogenesis of the virus has been obtained, these findings strongly suggested AiV-1 could be one of the agents causing human gastroenteritis.

*Aichivirus B* consists of two types, bovine kobuvirus 1 (Yamashita *et al.*, 2003) and sheep kobuvirus 1 (Reuter *et al.*, 2010a). The prototype strain of bovine kobuvirus 1 (BKV-1) type of the species *Aichivirus B*, named U-1, was isolated as a cytopathic contaminant in the culture medium of HeLa cells. *Aichivirus B* causes common infection with no confirmed disease and the virus is endemic among cattle worldwide (Reuter *et al.*, 2011).

*Aichivirus C* was accidentally discovered in 2008 in Hungary while screening faecal samples from pigs for calicivirus (norovirus and sapovirus) (Reuter *et al.*, 2008). *Aichivirus C* consists of a single type, porcine kobuvirus 1 (PKV-1). PKV-1 has often been detected at high frequency in faeces from healthy pigs, wild boars and diarrhoeic pigs and prevalence generally decreases with age of pigs (Chen *et al.*, 2013; Di Bartolo *et al.*, 2015; Khamrin *et al.*, 2010; Khamrin *et al.*, 2009; Park *et al.*, 2010; Reuter *et al.*, 2013; Ribeiro *et al.*, 2013). Some studies showed PKV infection was associated with diarrhoea in pigs (Barry *et al.*, 2011; Chen *et al.*, 2013; Park *et al.*, 2010). PKV RNA was also detected in serum of virus-infected pigs indicating the possibility of kobuvirus viraemia (Barry *et al.*, 2011; Fan *et al.*, 2013; Reuter *et al.*, 2010b). In summary, porcine kobuvirus is endemic and distributed worldwide.

#### **1.5.6. Hunnivirus**

Hunnivirus (previously known as Hungarovirus) was originally isolated in cell cultures from sheep in Northern Ireland (McFerran *et al.*, 1969). Subsequent characterization by electron microscopy (McFerran *et al.*, 1971), biochemistry (Adair *et al.*, 1987) and sequencing of the 450 nucleotides at the 3' end of the genome (Knowles, 2005) confirmed that this was a novel picornavirus. The virus was recognised as a new genus in the *Picornaviridae* family when the complete genome sequences of the first three genotypes from cattle and sheep in Hungary (Reuter *et al.*, 2012) and Northern Ireland (unpublished data) were determined. The virus name,

hunnivirus (HuV), is derived from these two countries, where the first three HuVs were found. The forth type was recently detected in rats in New York City (Firth *et al.*, 2014). The *Hunnivirus* genus is comprised of a species, *Hunnivirus A*, with four virus types (HuV-A1 to -A4). Interestingly, the nucleotide sequence and secondary RNA structure of the 5'UTR of HuVs are homologous to those of parechoviruses; the nucleotide sequences of the HuV IRES is particularly similar to those of HPeVs, especially HPeV-3, consistent with relatively recent modular exchange of this sequence between picornavirus genera. Meanwhile the amino acid sequences of the coding region are more closely related to those of porcine teschoviruses. The host range of HuVs and their pathogenicity remains unknown (Reuter *et al.*, 2012).

In Vietnam, a hot-spot for infectious diseases, among the newly discovered picornaviruses, only kobuviruses have been reported in river water (all 3 species) (Inaba *et al.*, 2014) and faecal specimens (members of *Aichivirus A*) (Pham *et al.*, 2007). This may be due to the lack of diagnostic tools and surveillance programs. The study hopes to learn more about their occurrence and potential disease associations.

#### **1.6. VIZIONS (Vietnam Initiative on Zoonotic Infections)**

There are still many gaps in the knowledge of emerging pathogens such as epidemiology of pathogens at the animal-human interface, drivers underlying pathogen emergence, the nature of the species barrier and the factors that enable pathogens to cross these barriers and establish transmission between humans (Woolhouse & Gaunt, 2007). These together with the recognition that most of new emerging infectious diseases in humans are zoonotic and that the human–animal interface is the key process of emergence have given rise to an urgent need to perform co-ordinated surveillance of humans who are highly exposed to animals (“at-risk” human populations) and animals populations to which people are exposed. Application of new approaches for pathogen detection and discovery, such as arrays, ultra-deep sequencing, complete genome sequencing, with traditional methods would be useful for this investigation. Despite agreement on the need for integrated surveillance approaches, there has been no such a global system and it is still far from reality (Hughes *et al.*, 2010). In response to this need, the Wellcome Trust funded project -

VIZIONS - is establishing a platform for cross-disciplinary research on human and animal health in Vietnam, a country regarded as a hot spot for emerging infectious diseases. Findings from the project will help understand the nature, public health importance and aetiology of the large number of undiagnosed infectious disease cases in Vietnam. Its hypothesis is that a significant number of these infections are caused by zoonotic pathogens which, in some cases, may include currently unrecognized and possibly newly emerging agents. The study will provide new insights into the genetic diversity of pathogens (with focus on viruses) in humans and animals and into factors (biological and behavioural processes) underlying the emergence of novel pathogens.

### **1.7. Aims of this thesis**

The rapid accumulation of data from viral metagenomics has resulted in a growing list of “orphan” human and animal picornaviruses whose pathogenicity and zoonotic potential, if any, remain unknown. Meanwhile, it is well established for picornaviruses that a few substitutions in the genome may remarkably change the virulence of a virus variant. This is illustrated by PV in which two nucleotide substitutions attenuate the Sabin 2 strain, and 10 substitutions are involved in attenuating the Sabin 3 strain (Kew *et al.*, 2005). Testing linkage between a new viral species and animal/human disease, therefore, includes an appreciation of the full range of genetic diversity of the viral species (Blinkova *et al.*, 2009). This thesis, as part of VIZIONS, hopes to address some gaps in our knowledge on molecular epidemiology, disease association, genetic diversity of picornaviruses and zoonotic source of picornavirus infection in humans. The main hypotheses are the studied picornaviruses may play a role in human and animal diseases, and the animals may harbour zoonotic picornaviruses. More specifically, studies in this thesis were carried out to answer the following questions:

- 1) How prevalent and diverse are picornaviruses in studied samples and in comparison with other regions of the world?
- 2) Are the identified viruses associated with disease and age of infected humans and animals?
- 3) Which viruses are circulating among different animal species and geographic-matched human populations?

#### 4) Which animals may be the sources of picornavirus infections in humans?

A large number of faecal samples from different animals (domestic pigs, wild boars, chickens, ducks, bats, rats, bamboo rats, civets, porcupines) that were in close and/or frequent contact with humans and from humans were used, as picornaviruses are mainly transmitted via faecal-oral route (Simonsson *et al.*, 2013).

One of the primary goals of this thesis was to understand the prevalence of the targeted picornaviruses in the studied samples. It may vary significantly between picornaviruses and between animal/human samples. The absence and presence of screened picornaviruses are presented and discussed in Chapter 3. Findings in this chapter are the background for subsequent studies in the next chapters on positive samples.

EVs and kobuviruses known to infect pigs and wild boars include CVB5, members of *EV-G* and *Aichivirus C*. Before this work, the species *EV-G* consisted of 7 types detected in wild boars, domestic pigs and sheep (<http://www.picornaviridae.com>, accessed October 15, 2013). Its genetic diversity had not been fully characterized and its pathogenicity was unknown. Chapter 4 focuses on the genetic characterization of EVs and kobuviruses infecting domestic pigs and wild boars by analysis of VP1 region and complete genome sequencing. Our findings of novel EV types, their recombination, and disease association of kobuvirus in pigs were published (Nguyen *et al.*, 2015 [Epub ahead of print]; Nguyen *et al.*, 2014a).

Some human EV types have been detected in non-human primates (NHPs), suggesting our close relatives may be a source of EV infection in humans (Harvala *et al.*, 2012; Harvala *et al.*, 2011b; Harvala *et al.*, 2014; Oberste *et al.*, 2013a; Oberste *et al.*, 2013b; Sadeuh-Mba *et al.*, 2014). Chapter 5 identified species and type distributions of EVs infecting Old World monkey species in Cameroon and their relationship to variants infecting local human populations. This is a larger scale continuation of previous studies of EV infection in African primates (Harvala *et al.*, 2012; Harvala *et al.*, 2011b; Harvala *et al.*, 2014) and fits within the remit of the PhD investigating zoonotic sources of human infections. The results showed the existence of novel EVs with chimeric

genome and we proposed new criteria for the division of EVs into genetically defined species (Nguyen *et al.*, 2014b).

Except for cardioviruses, which have been well studied in mice and rats, little was known about infection of kobuviruses and viruses of the new genus *Hunnivirus* in rats, especially bamboo rats and civets. This is of public health concern as these small rodents are a common source of food for the native peoples in Vietnam while a wide range of known and novel viruses from groups with important human pathogens such as sapoviruses, cardioviruses, kobuviruses, parechoviruses, rotaviruses and hepaciviruses were reported from rats (Firth *et al.*, 2014). Genetic characterization of these viruses in rats, bamboo rats and civets is the focus of Chapter 6.

Chapter 7 reveals picornavirus types detected in human samples from at-risk populations. The results indicated no overlaps between picornaviruses circulating in humans and animals in Vietnam.

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## Chapter 2. Materials and methods

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### 2.1. Biological samples

Sampling was performed by colleagues at the Oxford University Clinical Research Unit (OUCRU) - the leading partner of VIZIONS. Most of animal samples in this thesis were taken in provinces of Dong Thap in the Mekong delta and Dak Lak in the central highland (Table 2.1, Figure 2.1). Dong Thap is characterized by a high density of domestic pig and chicken farms, as well as duck flocks (Tu *et al.*, 2015). With an area of 3,238 km<sup>2</sup>, the province is home to about 1.6 million people, 274,000 pigs, 1.3 million chickens, 3.1 million ducks, and 22,000 cattle and buffalo (Carrique-Mas *et al.*, 2014). It is also notable for rice-field rats as a source of meat in Vietnam. Meanwhile, Dak Lak has a large number of farms raising wildlife animals including bamboo rats, civets, porcupines, deer and snakes for meat. These two provinces were thus targeted for sampling of human samples from high risk cohorts.

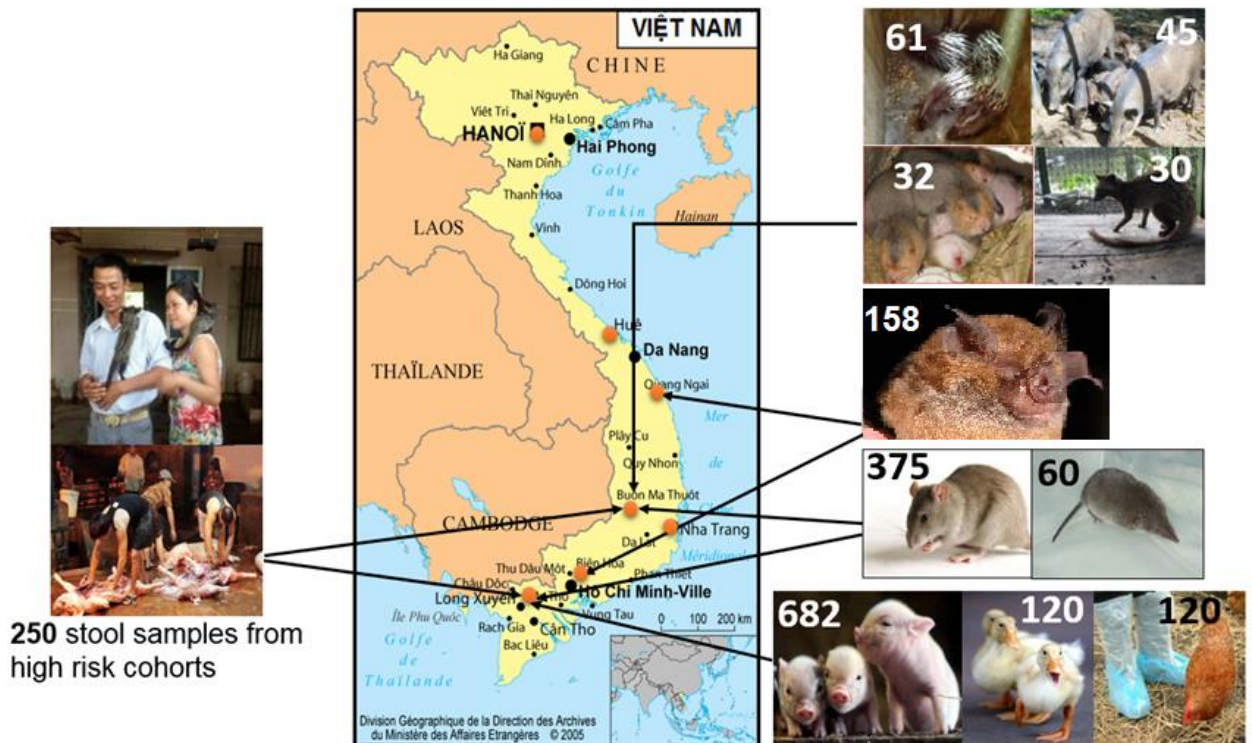
For all of the sampled animals in Vietnam, no previous prevalence of the targeted viruses had been reported before in Vietnam. Therefore, a prevalence of 50% was used for sample size calculation as recommended (<https://select-statistics.co.uk/calculators/sample-size-calculator-population-proportion/>, accessed February, 10, 2012). Calculations showed at least 383 samples were required to estimate the prevalence of viruses with a confidence level of 95% and an error margin of 5%. However, the limitation of sampling in this thesis was the insufficient sample sizes of live bats, shrews, civets and porcupines (Table 2.1).

**Table 2.1.** Origin and sampling periods of samples used in this thesis.

Sample origin	Number of samples	Collection provinces	Collection dates
Pig ( <i>Sus scrofa</i> )	682	Dong Thap	February – May 2012
Chicken (boot swab)	120	Dong Thap	February – May 2012
Duck (boot swab)	120	Dong Thap	February – May 2012
Rat (purchased)	150 (about 30 rats per province)	Dong Thap, Tien Giang, An Giang, Vinh Long and Can Tho	October 2012
	32	Dak Lak	April 2014
Rat (trapped)	125	Dong Thap	26 February – 8 March 2013
	100	Dak Lak	April – May 2014
Shrew ( <i>Suncus murinus</i> )	10	Dong Thap	26 February – 8 March 2013



Shrew ( <i>Suncus murinus</i> )	50	Dak Lak	April 2014
Farm-bred boar ( <i>Sus scrofa</i> )	45	Dak Lak	April 2014
Civet ( <i>Paradoxurus hermaphroditus</i> )	30	Dak Lak	April 2014
Porcupine ( <i>Hystrix brachyura</i> )	61	Dak Lak	April 2014
Bat	158	Quang Ngai, Dong Nai	May 2013
Monkey	113	Campo, Djoum, Ekom, Gribi, Lomie (Cameroon)	2006-2012
Human (high risk cohort)	250	Dong Thap, Dak Lak	March 2013 – September 2015



**Figure 2.1.** Sampling sites in Vietnam and sample numbers

### 2.1.1. Pigs and wild boars

Faecal samples were collected from 104 farms in four out of twelve districts (Cao Lanh, Chau Thanh, Hong Ngu and Thanh Binh) of Dong Thap province from February to May 2012. In these districts, a census of all registered farms was available. These randomly selected farms represented 3 farm size categories defined as: small (< 10 pigs), medium (10 – 50 pigs) and large (> 50 pigs). Approximately 9 farms per category per district were enrolled. At each farm, 10 freshly voided samples (faeces found in the pen within 5 minutes after excretion, about 5 g per sample) from randomly

selected healthy pigs and up to four samples from pigs with diarrhoea were included. Faecal samples were recorded as diarrhoeic or not based on their consistency. From that collection, a subsample of 682 samples, including all 89 diarrhoeic samples and representing all sampled farms (5 – 7 samples / farm), were used in this study. In addition, 45 faecal samples collected in April 2014 from healthy wild boars which were kept at natural living conditions in 6 farms in Dak Lak province were also included.

### **2.1.2. Chickens and ducks**

The survey was conducted in the same four districts over the same period with sampling of pigs in Dong Thap province mentioned above. The farm sizes were determined on the basis of animal numbers per farm: for chickens 20 – 50 (small), 51 – 100 (medium) and > 100 (large); for ducks 50 – 200 (small), 201 – 1000 (medium) and > 1000 (large). 120 chicken and 120 duck farms were randomly selected from the census of registered farms.

In addition to individual samples (10 / farm), environmental sampling was performed using boot swabs to collect naturally pooled faeces. The number of boot swabs collected depended on farm sizes: 3, 4 and 5 boot swabs for small, medium and large farms, respectively. Briefly, farms' areas dedicated to housing chickens or ducks were divided into equally sized sections. Then boot swabs were taken from each section by walking over 50 steps while wearing a pair of sterile, pre-moistened overshoe protectors. Shuffling motions were focused on areas where fresh droppings were visible. Where boot swab sampling was not possible on housing areas (i.e. chickens in cages or stilt houses), faeces from 10 sampling points were taken using hand-held gauze swabs (~25 g per swab). Each pair of swabs per section were placed into a sterile tube containing 15ml of PBS. After that 1ml of mixed sample was preserved in 1ml of RNAlater (Qiagen, UK). Since one pair of boot swabs is roughly equivalent to sampling 60 individual droppings (Skov *et al.*, 1999), this sampling strategy helped to sample a constant proportion of animals from each farm (Tu *et al.*, 2015). In this thesis, boot swabs were used for initial screening for picornaviruses. If positive, individual

samples from those farms were screened separately. Aliquots of all swabs from each farm were pooled prior to nucleic acid extraction.

### 2.1.3. Rats and shrews

150 rats and 32 bamboo rats were purchased from markets in five of twelve provinces in the Mekong Delta (about 30 rats / province) in October, 2012 and from a market in Dak Lak in April, 2014, respectively. Rat trapping was carried out in different locations (pig and poultry farms, rice fields, fruit groves, tropical forests, markets, slaughter-house) in the provinces of Dong Thap during March 2013 and Dak Lak in April 2014 (which was the only sampling trip I participated in). A total of 30 lines (one per location) of 10 Tomahawk live traps (<http://www.livetraps.com>) were set up, checked for trapped rats, and reset over 10 consecutive mornings. Traps with rat(s) trapped inside were brought back to the laboratory and replaced by new traps. In addition to 225 trapped rats, 60 shrews (*Suncus murinus*) were accidentally trapped and sampled. Rats and shrews were euthanized by an overdose of inhalant isoflurane using the American Veterinary Medical Association guidelines (available at <https://www.avma.org/KB/Policies/Documents/euthanasia.pdf>). After that, biological and morphometric characteristics (weight, sex, body length, tail length, foot length, ear length, skull length, ear length, fur colour, reproductive status) were measured and recorded to allow species identification. Faecal samples as well as other samples (e.g. liver, spleen, lung) were aseptically collected during post-mortem and stored in sterile tubes at -20°C. Special precautions were taken to avoid cross-contamination.

**Table 2.2.** Species of rats and shrews

Species	Number of sample
<i>Rhizomys pruinosus</i>	32
<i>Rattus norvegicus</i>	40
<i>Bandicota indica</i>	65
<i>Rattus argentiventer</i>	104
<i>Rattus tanezumi</i>	80
<i>Rattus exulans</i>	76
<i>Rattus nitidus</i>	10
<i>Suncus murinus</i>	60

### 2.1.4. Bats

A total of 158 bats were trapped at 6 sites in the provinces of Dong Nai (in Cat Tien National Park) and Quang Ngai in May 2013. Bats were trapped by using mist nets and harp traps set at ground level. Traps were checked for trapped bats, and reset over 10 consecutive mornings. Species of the trapped bats (Table 2.3) were identified by morphology to make sure they were out of the IUCN (International Union for Conservation of Nature) Red List of threatened species (available at <http://www.iucnredlist.org/>). Trapped bats were euthanized and samples were collected as described above for rats and shrews.

**Table 2.3.** Species of the trapped bats

Species	Number of bats
<i>Cynopterus sphinx</i>	14
<i>Glischopus bucephalus</i>	2
<i>Hipposideros cineracea</i>	1
<i>Hipposideros armiger</i>	6
<i>Hipposideros cineracea</i>	3
<i>Hipposideros galeritus</i>	4
<i>Hipposideros larvatus</i>	7
<i>Hipposideros pomona</i>	3
<i>Kerivoula hardwickii</i>	4
<i>Kerivoula kachinensis</i>	2
<i>Megaderma spasma</i>	4
<i>Megaderma lyra</i>	1
<i>Megaerops niphanae</i>	3
<i>Murina annamitica</i>	1
<i>Murina cyclotis</i>	1
<i>Murina eleryi</i>	1
<i>Murina walstoni</i>	1
<i>Myotis muricola</i>	10
<i>Myotis rosette</i>	6
<i>Pipistrellus sp.</i>	1
<i>Rhinolophus acuminatus</i>	25
<i>Rhinolophus affinis</i>	23
<i>Rhinolophus chaseli</i>	3
<i>Rhinolophus lepidus</i>	2
<i>Rhinolophus luctus</i>	1
<i>Rhinolophus pusillus</i>	12
<i>Rhinolophus shameli</i>	1
<i>Rhinolophus sinicus</i>	9
<i>Scotomanes ornatus</i>	1
<i>Scotophilus heathi</i>	6

#### 2.1.5. Civets and porcupines

Civets and porcupines are commonly raised in Dak Lak for civet coffee production and meat. 30 civets and 61 porcupines were purchased from 4 and 8 randomly selected

farms raising these animals, respectively. They were euthanized and samples were taken as described above for rats and shrews.

#### **2.1.6. Monkeys**

This was a separate study which continued previous work investigating EVs in wild NHPs in Cameroon (Harvala *et al.*, 2011b; 2014). A total of 113 monkey stool samples collected from 8 forest sites in the southern part of Cameroon over the period of 2006 – 2012 were included in this study. Sampling was performed primarily around night nests or feeding sites from natural habitat areas of NHPs - remote and sparsely populated areas with minimal human contact (Keele *et al.*, 2006). Samples were collected by experienced trackers, preserved in RNAlater (Ambion, USA), stored at room temperature at base camps for a maximum of 3 weeks and subsequently transported to a central laboratory in Yaoundé before shipment to Edinburgh.

#### **2.1.7. Humans**

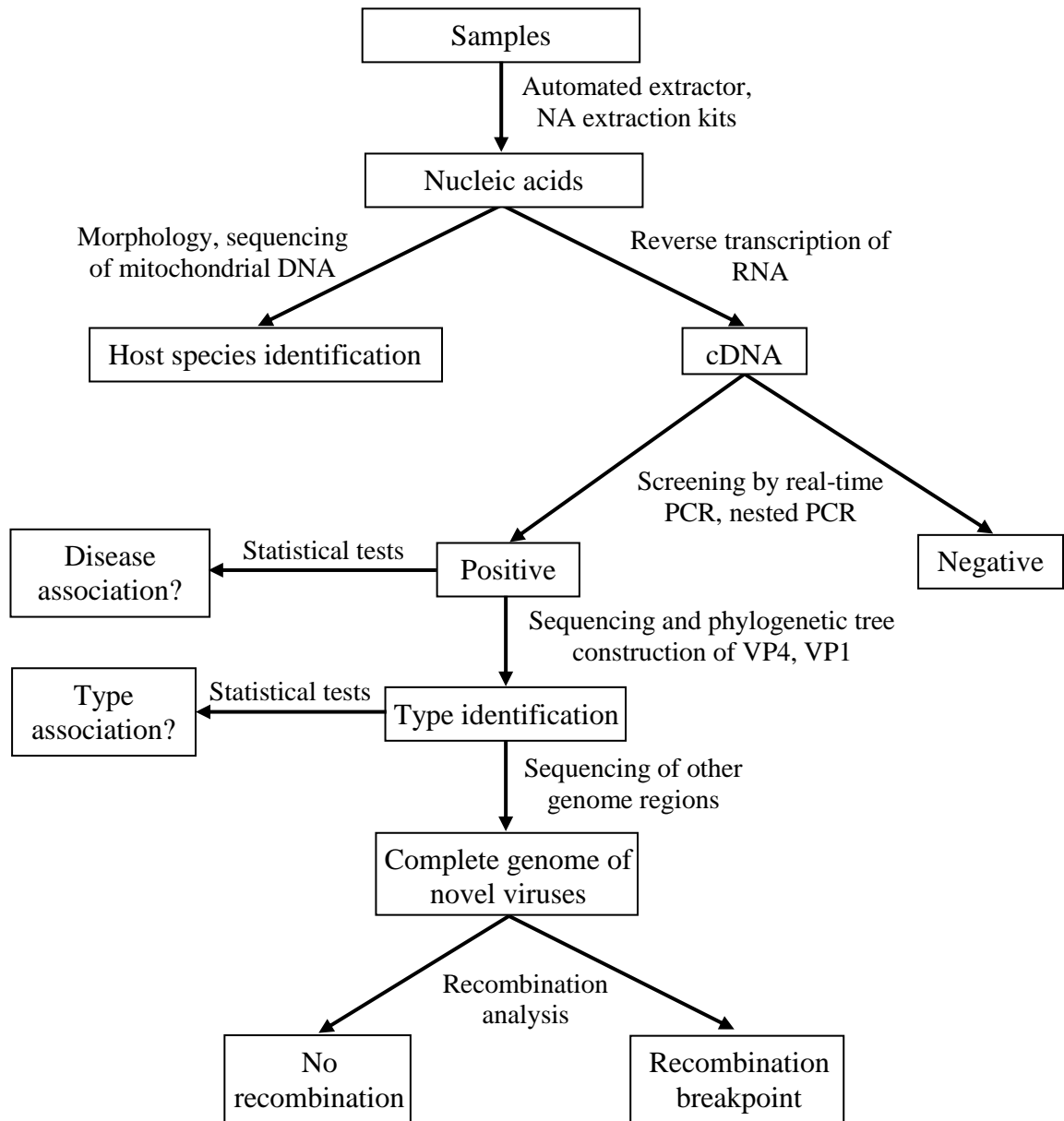
Human samples used in this thesis included 250 faecal samples from cohorts at high risk due to occupational exposure to animals in Dong Thap (100 samples) and Dak Lak (150 samples). These samples were collected mostly from people with frequent contact with animals such as farmers raising pigs and/or chickens, ducks, slaughter-house workers, animal health workers.

### **2.2. Laboratory techniques**

Nucleic acid extraction, PCR and DNA sequencing are the key laboratory techniques involved in this thesis (Figure 2.2). Considerable precautions were taken in order to prevent PCR contamination due to its high sensitivity. Central to these precautions was the implementation of lab standard operating procedures (SOPs) which provided step-by-step instructions and detailed lab spaces for each step. In brief, nucleic acid extraction, master mix preparation, cDNA synthesis, DNA amplification and gel electrophoresis were carried out in dedicated and separated lab areas. Each work area had lab coats and gloves. In addition, reagents and primers for reverse transcription and PCR were kept in small aliquots in dedicated, clean areas where master mixes

were prepared. Templates (RNA, DNA) were added to PCR tubes containing the reaction mixtures after all other reagents (Kwok & Higuchi, 1989). A negative control using the same reagent mix with actual samples was always included in experiments to detect potential contamination.

Reverse transcription of RNA, PCR and sequencing were performed in thermal cyclers (Thermo Scientific, UK).



**Figure 2.2.** The flowchart of key steps and methods used in this thesis.

### 2.2.1. Nucleic acid extraction

Screening of VIZIONS samples for targeted picornaviruses was performed at OUCRU in Ho Chi Minh City, Vietnam. About 10% (w/v) faecal suspensions in phosphate buffered saline (PBS) were mixed thoroughly and clarified by low-speed centrifugation. Nucleic acids were directly extracted from 180 µl of the resulting supernatant using MagNA Pure 96 Viral NA small volume kit and an automated extractor (Roche, Basel, Switzerland). RNA quality and the presence of PCR inhibitors was assessed by spiking samples with 20 µl of an RNA internal extraction control (EAV - equine arterivirus) (Scheltinga *et al.*, 2005) prior to extraction. EAV was kindly provided by OUCRU staff. The principle steps are:

- The sample material and internal extraction control are lysed, nucleic acids are released and nucleases are denatured.
- The nucleic acids bind to the silica surface of the added magnetic glass particles (MGPs), due to the chaotropic salt conditions and the high ionic strength of the Lysis/Binding Buffer.
- MGPs with bound nucleic acids are magnetically separated from the residual lysed sample.
- Washing steps remove unbound substances (proteins, cell debris, PCR inhibitors, etc.).
- Purified nucleic acids are eluted from the MGPs in 50 µl of elution buffer and stored at -80°C until use.

RNA of positive samples was re-extracted at OUCRU from the original specimens using QIAamp viral RNA mini kits (Qiagen, UK) and sent on dry extraction columns to Edinburgh for further genetic characterization. Briefly, 140 µl of sample was mixed with 560 µl of prepared viral lysis buffer (Buffer AVL) containing 1 µg/µl carrier RNA and incubated at room temperature for 10 minutes. The added carrier RNA enhances the yield of viral RNA during extraction by reducing degradation of viral nucleic acid due to residual RNases and by assisting the binding of RNA to the QIAamp Mini column membrane. 560 µl of ethanol (96 – 100%) was added and mixed by pulse vortexing. The resulting solution was passed through a QIAamp Mini column at 6,000 g (8,000 rpm) for 1 minute. Each column was then subjected to washing with wash

buffer 1 (Buffer AW1), followed by wash buffer 2 (Buffer AW2). Residual buffer was removed by centrifugation at full speed for 1 minute. The dry extraction columns from this step were sent to Edinburgh and RNA was then eluted in 60 µl of nuclease free water. Extracted RNA was either used in PCR immediately or stored at -20°C.

For species identification of monkeys by mitochondrial sequencing, faecal DNA was extracted using the QIAamp Stool DNA Mini kit (Qiagen, US) according to the instructions from the manufacturer.

### 2.2.2. Reverse transcription of RNA

Two-step RT-PCR protocols were used in the majority of studies. Single stranded cDNA was synthesized by reverse transcription (RT) to allow amplification of extracted viral RNA by PCR. RT was carried out using the SuperScript III Reverse Transcriptase (Invitrogen, UK) according to the manufacturer's instructions. Briefly, 6 µl of eluate obtained from RNA extraction was incubated with 1 µl (100 ng) of random hexamer primers, 1 µl of 10 mM dNTP mix (Life Technologies, UK) and 5 µl nuclease free water at 65°C for 5 minutes, then chilled on ice for at least 1 minute. 7 µl of master mix containing RT (Table 2.4) was added to each sample. The reaction mixture was then incubated at 25°C for 5 minutes followed by an elongation step at 50°C for 60 minutes. Finally, the reaction was heated at 70°C for 15 minutes to inactivate the reverse transcriptase enzyme and prevent it binding to the single stranded cDNA. cDNA was then either used immediately for PCR reactions or stored at -20°C.

All positive and negative controls used in downstream PCR were included in the RT reaction to ensure consistency of results. The use of random hexamer primers in the RT step enabled synthesized cDNA to be used both for screening of different picornaviruses and the amplification of multiple genome regions.

**Table 2.4.** RT master mix.

4 µl	5X First-Strand Buffer <sup>a</sup>
1 µl	0.1 M DTT
1 µl	RNaseOUT™ (40 units/µl) <sup>b</sup>
1 µl	SuperScript™ III RT (200 units/µl)

<sup>a</sup> 5X First-Strand Buffer is supplied with the Superscript III RT kit and contains 250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl<sub>2</sub>.



<sup>b</sup> Recombinant RNase Inhibitor (Invitrogen, UK)

### **2.2.3. Polymerase chain reaction (PCR) and related techniques**

#### **2.2.3.1. Design of oligonucleotide primers**

Primers previously unavailable in literature were designed for use in the amplification of various picornavirus genome regions. All of the designed primers, including degenerate ones to overcome high genetic diversity within regions, must be specific to avoid amplification of non-target sequences. Each primer set was tested for sensitivity using serial 10-fold dilutions of known positive samples.

All relevant sequences were first downloaded from GenBank. After alignment using SSE version 1.1 (Simmonds, 2012), these sequences were examined for highly conserved areas around the target regions. Areas of high sequence conservation were then analysed for melting temperature ( $T_m$ ), hairpin formation, self-annealing, hetero-dimer by the Oligo Analyzer Tool (available at <http://www.idtdna.com/calc/-analyzer>). Short fragments were considered for the PCR primers in fulfillment of the following criteria:

- Length of 18 – 30 nucleotides
- Predicted  $T_m$  close to 60°C
- High sequence conservation (as few degenerate bases as possible)
- G+C content between 40 – 60% with a G or C at the 3' end where possible
- No runs of 4 or more of one base, or dinucleotide repeats
- No significant self-annealing ( $\Delta G \geq -10$  kcal/mol) (Bustin & Nolan, 2013) or hairpin formation where possible

Primer pairs for use in the same PCR reactions were selected to have no significant hetero-dimer formation and  $T_m$  differences of no more than 5 degrees, where possible. All primers were blasted ([www.ncbi.nlm.nih.gov/tools/primer-blast](http://www.ncbi.nlm.nih.gov/tools/primer-blast)) to ensure the specificity of each primer set.

**Table 2.5.** Codes for nucleotides used in primer design.

Base code	Nucleotides
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
K	G or T
M	A or C
R	G or A
S	G or C
W	A or T
Y	T or C
B	G, C or T
D	G, A or T
H	A, C or T
V	G, C or A
N	A, C, G or T

### 2.2.3.2. PCR protocols

Nested PCR was used for virus detection (EVs in monkeys, cosaviruses, cardioviruses, kobuviruses and HuVs) and characterization of all viruses in positive samples. Positive controls for the screened viruses mentioned above were usually from clinical specimens or concentrated sewage (cosavirus). The reaction mixture (Table 2.6) was subjected to a basic PCR protocol comprised an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of denaturation (94°C for 30 s), annealing (50°C for 30 s) and elongation (72°C, 60 s/kb). Final extension was at 72°C for 5 minutes. After that, 1 µl of the first-round reaction was used for the second-round PCR with similar conditions.

**Table 2.6.** Reagents used in PCR reactions

5X colorless or green GoTaq buffer <sup>a</sup>	4 µl
Sense primer (10 µM)	1 µl
Antisense primer (10 µM)	1 µl
3 mM dNTPs <sup>b</sup>	0.2 µl
GoTaq DNA polymerase (Promega, UK) (5 u/µl)	0.1 µl
Either cDNA or first round PCR product <sup>c</sup>	1 – 2 µl
Nuclease free water	to 20 µl

<sup>a</sup> The 5X colourless and green GoTaq buffers were used for the first- and second-round PCR, respectively. The green buffer increases the density of the sample, allowing PCR products to be loaded directly onto gels without loading dye. Both buffers contain MgCl<sub>2</sub> at a concentration of 7.5 mM.

<sup>b</sup> dNTPs (Life Technologies, UK) were acquired as a mix of four nucleotides (dATP, dCTP, dGTP, dTTP), each at a concentration of 10 mM and were then diluted to make 3 mM stocks for use in PCR.

<sup>c</sup> 2 µl of cDNA was used for the first round of PCR. 1 µl of first round product was used for the second round of PCR.

For screening, the prevalence of viruses in a subset of 50 randomly selected samples was initially checked. After that, cDNA of the remaining samples was screened either separately for viruses with high prevalence or in pools of 5 samples for viruses with low prevalence. Screening of EV in domestic pigs was completed by a technician of OUCRU. Results were kindly provided with permission.

Individual PCR protocols with new primer sets were optimised prior to use with study samples. The optimal set of conditions for each PCR reaction was determined after varying certain parameters (annealing temperature, primer, MgCl<sub>2</sub> and dNTP concentrations). The sensitivity of several PCR protocols was remarkably improved when one of the same primers was used in both rounds (hemi-nested reactions). Specific primers for each PCR reaction with modifications (if any) to the basic protocol are detailed in Table 2.7 together with which thesis chapter these reactions relate to.

#### **2.2.3.3. Real-time PCR**

Real-time PCR was used to either screen for viruses (EV, parechovirus) or measure viral loads (EV in monkeys, PKV in domestic pigs) in samples positive by nested PCR. Published protocols for EV (Dierssen *et al.*, 2008) and parechovirus (Benschop *et al.*, 2008a) were used. Positive controls were 10-fold dilution series (10<sup>6</sup> to 10<sup>0</sup> copies/μl) of CAV-16 and HPeV-1 RNA transcripts available in the lab for use (McLeish *et al.*, 2012). RNA was diluted in RNA storage solution (Ambion, UK). In order to increase the stability of RNA transcripts, the storage solution was added with 0.05 μg/ml herring sperm carrier RNA and 0.1 U/ml RNasin (New England BioLabs, UK). Detection limits of 100 and 75 copies of EV and HPeV cDNA per reaction respectively were obtained as in published papers (Benschop *et al.*, 2008a; Dierssen *et al.*, 2008).

**Table 2.7.** List of primers (Invitrogen, UK) and probes (IDT, UK).

<b>Primers and probes for real-time PCR</b>					
<b>Virus</b>	<b>Target region</b>	<b>Oligo sequence (5' – 3')</b>		<b>Chapter</b>	<b>Source</b>
EV	5'UTR	Sense	CCCTGAATGCGGCTAAT	3	(Beld <i>et al.</i> , 2004)
		Antisense	ATTGTCACCATAAGCAGCC		
		Probe	6-FAM-CGGAACCGACTACTTTGGGT-BHQ1		
EV	5'UTR	Sense	ACATGGTGTGAAGAGTCTATTGAGCT	5	(Dierssen <i>et al.</i> , 2008)
		Antisense	CCAAAGTAGTCGGTTCCGC		
		Probe	6-FAM-TCCGGCCCCTGAATGCGGCTAAT-BHQ1		
HPeV	5'UTR	Sense	CTGGGGCCAAAAGCCA	3	(Benschop <i>et al.</i> , 2008a)
		Antisense	GGTACCTTCTGGGCATCCTTC		
		Probe	LCRED610-AAACACTAGTTGTAWGGCCC-BHQ1		
LV	5'UTR	Sense	GCGGTCCCCTCTTCACAG	3	(Mantke <i>et al.</i> , 2007)
		Antisense	GCCCAGAGGCTAGTGTTACCA		
		Probe	6-FAM-TGTC(C/G)AGAGGTGAAAGC-MGB		
PKV	5'UTR	Sense	CGTGCTGAGTAATGGGATAGGAGAT	4	This thesis
		Antisense	ACTCTATCAAGCAGTACATGGTCAAG		
		Probe	6-FAM-TGGATTCCC/ZEN/AGCCACGCAACCACAG-IBFQ		
EAV	ORF1a	Sense	CATCTCTTGCTTTGCTCCTTAG	3	(Scheltinga <i>et al.</i> , 2005)
		Antisense	AGCCGCACCTTCACATTG		
		Probe	CY5-CGCGCTCGCTGTCAGAACAAACATTATTGCCACAGCGCG-BHQ2		

Reporter dyes: carboxy-fluorescein (FAM) and indodicarbocyanine (Cy5). Quenchers: black hole quencher (BHQ) 1 and BHQ2, Iowa black FQ (IBFQ), and ZEN.

Other primers for screening						
Virus	Region	Primer	Sequence (5' – 3')	Modifications to basic reagents, cycling conditions	Chapter	Source
EV (in monkeys only)	5'UTR	309s	TGTAGHTYWGGTCGATGAGTC	Hemi-nested reaction - same sense primer in both rounds	3	This thesis
		573oa	RGAAACACGGACACCCAAAGTAGT			
		563ia	ACACCCAAAGTAGTYGGTYCCR			
Cosavirus	5'UTR	os	CGTGCTTTACACGGTTTTTGA	Annealing temperature of 55°C in both rounds	3	(Kapoor <i>et al.</i> , 2008)
		oa	GGTACCTTCAGGACATCTTTGG			
		is	ACGGTTTTTGAACCCACAC			
		ia	GTCCTTTCGGACAGGGCTTT			
Cardiovirus	5'UTR	530os	GGYCKAAGCCGCTYGGAATAHG	Annealing temperature of 55°C in both rounds	3	This thesis
		969oa	GGKCYCCWGRTCAGATCCC			
		777is	TGGYRACARGWGCCTCTGCG			
		937ia	GGGTACCTTCWGGRCATYCTTSR			
Kobuvirus	3D	7185os	GGTGGWCTYATHGASTACATGC	Annealing temperature of 55°C in both rounds	3	Dr. Colin Sharp, Roslin Institute, University of Edinburgh
		7807oa	GTGTCNGGRTCCATSACHGGGTG			
		7309is	TGGAYTACAAGTGYYTTTGATGC			
		7516ia	ATGATGGTGTTRAKGATRGARGTG			
HuV	5'UTR	396os	AGCCTGYGGAMYAACAACKGTAAC	Annealing temperature of 57°C in the 1 <sup>st</sup> round, 55°C in the 2 <sup>nd</sup> round	3	This thesis
		956oa	CAAAGAAAAAKTCTTCTG BCG TCTGACC			
		450is	TTWACAGCACCTMMTRGTTTGAT			
		840ia	GCCTCCATATTGMCTSYTGyTGKATTTG			

Primers for typing						
Virus	Region	Primer	Sequence (5' – 3')	Modifications to basic reagents, cycling conditions	Chapter	Source
EV	VP4	458os	CCGGCCCCTGAATGYGGCTAA	None	4, 5, 7	(Wisdom <i>et al.</i> , 2009)
		1125oa	ACATRTTYTSNCCAAANAYDCCCAT			
		547is	ACCRACACTTTGGGTGTCCGTG			
		1087ia	TCWGGHARYTTCCAMCACCANCC			
EV-A	VP1	2268os	CCNTGGATHAGYAAACACNCAIT	None	7	(Leitch <i>et al.</i> , 2009)
		3109oa	GGRTANCCRTCRTARAACCAITG			
		2332is	TNASNATYTGGTAYCARACNAAYT			
		3016ia	GANGGRTTNGTNGKNGTYTGCCA			
EV-B	VP1	2293os	GGYTAYATNACNTGYTGGTAYCARAC	Annealing temperatures of 45°C in the 1 <sup>st</sup> round, 48°C in the 2 <sup>nd</sup> round	7	(Leitch <i>et al.</i> , 2009)
		3444oa	GGTGCTCACTAGGAGGTCYCTRTRTARTCYTCCCA			
		2361is	CTTGCTGCTTTGTGTGCGGCRGTGYAAYGAYTTYTCWG			
		3416ia	TCYTCCCACACRCAVTTYTGCCARTC			
SA5	VP1	2606os	CCAGCATTAACWGCARCRGAAACWGG	None	5	(Harvala <i>et al.</i> , 2011b)
		3287oa	TGGRTMCCAAGACCACCRCG			
		2650is	ACCAAGTGACACYRTRCAAACCAGG			
		3118ia	CCACCCRTCATARAACRWRCTATACGC			
EV-H	VP1	2098os	GGCAATAGAGARGAGGCAATGCTRGG	None	5	(Nguyen <i>et al.</i> , 2014b)
		3079oa	ATTRGCATTTGACACAAAAGGCAYKGA			
		2265is	CTTGTTYCAAACRAAYATGGTRGTTC			
		3021ia	GARGGRTTYGTYGARGATTGCCA			
EV-H	VP1	2841os	AYGTRCGCTTTGAYCTTGARGTSA	None	5	(Nguyen <i>et al.</i> , 2014b)
		3755oa	CGTGNGTTTGGTATCTCKCWGGRTAG			
		2993is	TGGCAATCYTCRACRAAYCCYTC			
		3741ia	TCTCTCAGGGTAGTAYTCGCTCGC			
EV_unassigned	VP1	2337os	TGTGGTAYCARACAAAYTTTGTRGTYCC	Annealing temperature of 57°C in both rounds	5	This thesis
		3526oa	GTCACAMCCVTGKGCRGTRGTG			
		2401is	GCGGCWGCWCARCCHAAYTT			
		3494ia	AGTAGRTCYCTYTCRTARCTCTCCCA			

Primers for typing						
Virus	Region	Primer	Sequence (5' – 3')	Modifications to basic reagents, cycling conditions	Chapter	Source
<i>EV-G</i>	VP1	2313os	GCTGGKTATRTKACYGGDTGGTWY C	Annealing temperature of 55°C in both rounds	4	(Nguyen <i>et al.</i> , 2014a)
		3421oa	TCTTCCCAATCDASRTTBTCCTCA			
		2395is	TGGCWGCACARCCNAAATTYTC			
		3390ia	GGGTGCAAGRKGCTRTTCA			
Cardiovirus	VP4	915os	CCAAGRATGYCCWGRAGGTACCC	Annealing temperature of 55°C in both rounds	6	This thesis
		1875oa	GCGTGRAAYTGDGANGCRTTRCA			
		947is	GTRHGGGATCTGAYCWGGRGMC			
		1596ia	ACAGTWGAYTGDGTGTTTGTRRCCG			
Kobuvirus	VP0	1278os	GTGCCAAYGGHTGGDCYCC	Annealing temperature of 55°C in both rounds	4	This thesis
		2028oa	CCTGCATGGAAATGWGWRCRTT			
		1311is	GCCTTGWWGATGSHCCYGTTC			
		1996ia	CTGGACDCGCCADCCRCARTT			
<i>Aichivirus A</i>	VP1	2794os	CCATYCCCTTCATYTCYAACTCCTACTGG	Annealing temperature of 58°C in both rounds	7	This thesis
		4044oa	CGAAGTGGGTRCAGTTGTTGGTKG			
		2867is	CGGCTAYGTYTCCATCTGGGATKC			
		3993ia	GGAGTTCAGCRAGRGTCCAATGK			
<i>Aichivirus C</i>	VP1	2817os	TACTGGAARGTCACYAAYATGGCYAAYCAG	Annealing temperature of 58°C in both rounds	4	This thesis
		4044oa	CAAAGTGRGTRCAGTTRTRGTRGCAGAG			
		2884is	GGGTGATGAAYCCRCTYACYGG			
		3936ia	GGTCYTCRTAVGCAACGTADGCG			
HuV	VP1	1580os	GGTGTBTTYWTRGTGCCMCAGTTTGT	Annealing temperature of 58°C in both rounds	6	This thesis
		3660oa	CCAATCACDGAAGRAGRAAAGCAG			
		2453is	AGTGGTGGSTATTYACAATTTGGC			
		3603ia	CGGGAAAGAATCACASCATARGAAAG			

Self-designed primers for complete genome of new EV types: EV-G8, EV-G9 (used in addition to EVG VP4, VP1 primers above)				
Region	Primer	Sequence (5' – 3')	Modifications to basic reagents, cycling conditions	Chapter
94 – 779	2os	TWAAACAGYCWGBGGGTGT	None	4
	1087oa	TCWGGHARYTTCCAMCACCANCC		
	94is	TAGTACCTTTGTACGCCTGTTTT		
	779ia	TTYTTRCTCATTTGCATHCCCAT		
571 – 2965	482os	CCGGCCCCTGAATGYGGCTAA	SuperScript III one step RT-PCR and SequalPrep long PCR were used.	4
	3390oa	GGGTKGCAAGRKGYCTRITCA		
	571is	ACCRACACTTTGGGTGTCCGTG		
	2965ia	GGRTTWGCKGCASTTTGCCA		
3187 – 5419	2313os	GCTGGKTATRTKACYGGDTGGTWYC	SuperScript III one step RT-PCR and SequalPrep long PCR were used.	4
	5806oa	GGRAAGTTRTACATCATDGTKCG		
	3187is (EV-G8)	TGCAAGCCAAAACAYATTAAAG		
	2679is (EV-G9)	GTGGAGAGCTTTTATTCTAGATC		
	5419ia	TTCATCATBCCDACNCCAAARTCCAT		
5187 – 7373	4835os	GAGAATAAYACCTTGAATGTGACAG	<ul style="list-style-type: none"> <li>- SuperScript III one step RT-PCR and SequalPrep long PCR were used.</li> <li>- Annealing temperature of 53°C in both rounds</li> </ul>	4
	7385oa	CATCCGGTGGGWGTRTTG		
	5189is	GAGATYCCAGTAGATGAGAGRA		
	7373ia	GTATTGHATCCAATTGAAGYTTWGG		



Self-designed primers for complete genome of novel EVs in Mandrill monkeys (used in addition to EV VP4, VP1 typing primers)					
Virus	Region	Primer	Sequence (5' – 3')	Modifications to basic reagents, cycling conditions	Chapter
EV_unassigned	1 – 440	1s	TTAAAATAGCCTSWGGGTTGYTCC	Hemi-nested reaction - same sense primer in both rounds	5
		440ia	GCTCARTAGGCTCTTCACACCATGTC		
		573oa	RGAAACACGGACACCCAAAGTAGT		
EV_unassigned	309 – 764	309s	TGTAGHTYWGGTCGATGAGTC	Hemi-nested reaction - same sense primer in both rounds	5
		764ia	TGTCKTGAMACYTGTGCTCCCAT		
		891oa	TGGGTCCTGGCTRAARTCYTGTTT		
CPML8109	1096 – 2486	1039os	ATTGTAGTAGGGTAYGGWGARTGGC	Annealing temperature of 58°C in both rounds	5
		2583oa	TGTGCCAATGTTATGGGAGGATGC		
		1096is	GTGGATAAACCAACCAARCCHGAYGT		
		2486ia	GTCCCCTTGACAGTACAGATGCTT		
CPML3961	1096 – 2496	1041os	GGTTGTGGCKTAYGGTGARTGGC	Annealing temperature of 58°C in both rounds	5
		2579oa	GCAATGTTGTGYGTRCTTTCCARTG		
		1096is	GTAGATAAACCAACRMARCCYAYGT		
		2496ia	GCCTACTGGRTCTCCTTGKAGAAATG		
GR2815	1096 – 2402	1040os	ATTGTTGTAGGGTATGGTGAGTGGC	Annealing temperature of 60°C in both rounds	5
		2441oa	TCAGCAAACGGACACTAAAGTTGGG		
		1096is	GTGGATAAACCAACCAARCCHGAYGT		
		2402ia	GCAATTATATATCCGTTGTTAGGTGCCCC		
EV_unassigned	3394 – 4826	3349os	AACAACWGGRGYKTTTGACAACA	Annealing temperature of 58°C in both rounds	5
		4956oa	CAGKGTGTTGAARTTYGCTGGRTCACA		
		3394is	GGWGC MRHTATGTGGGTAATTACAGA		
		4826ia	GTGCTCTGCTATCTGARATGGTKGG		
EV_unassigned	4692 – 6097	4447os	CGTACAGTTCAAGTCCAAATGCCGC	Annealing temperature of 60°C in both rounds	5
		6134oa	TCCWCCTTGAGTCTGGGTCTTTTC		
		4692is	GGTGTCTYAGTGTGAYTTYGTGCCA		
		6097ia	CGGGTTCCTTCACACCTTCAAAGAT		
EV_unassigned	5907 – 7466	5825os	CACAGGATGYTHATGTAYAAYTTYCCMAC	- Hemi-nested reaction - same antisense primer in both rounds - Annealing temperature of 55°C in both rounds	5
		5907is	TTCATGTTGGWGGYAATGGRCAYC		
		7466a	CACTCTGGTTGTGCTAAATTTWCCCCTG		

Self-designed primers for complete genome of novel HuV (used in addition to HuV VP1 primers above)				
Region	Primer	Sequence (5' – 3')	Modifications to basic reagents, cycling conditions	Chapter
74 – 840	52os	AAGARCCCTCACCTGTCA	Annealing temperature of 53°C in both rounds	6
	956oa	CAAAGAAAAAKTCTTCTGBCGTCTGACC		
	74is	GGGACCACCACTTTCAGT		
	840ia	GCCTCCATATTGMCTSYTGYTGKATTTG		
525 – 1605	525s	TTGGTATCAYGGCATAACCGKAGAG	Hemi-nested reaction - same sense primer in both rounds	6
	2673oa	ACATTYTCATTBGTRTTYTCWGCT		
	1605ia	ACAAACTGKGGCACYAWRAAVACACC		
812 – 2658	525os	TTGGTATCAYGGCATAACCGKAGAG	- SuperScript III one step RT-PCR was used for cDNA synthesis and the 1 <sup>st</sup> round PCR - Annealing temperature of 55°C in both rounds	6
	2673oa	ACATTYTCATTBGTRTTYTCWGCT		
	812is	CAAATMCARCARSAGKCAATATGGAGGC		
	2658ia	CATCTGAACTCAAARTCYTCACCAGC		
3455 – 5766	3391os*	GGAACCAGACYTDGATGARATGTT	- SuperScript III one step RT-PCR and SequalPrep long PCR were used. - Annealing temperature of 55°C in both rounds	6
	5886oa	GTCCCYAAGCTCTCTGCRYAKAAACATA		
	3455is	ACAGAYTTTGCRYACYTGGATTGAT		
	5766ia	ATGTCTCTGAAYTGYCTBCCTG		
5428 – 7577	5128os	GAAGGAHGTGARAARTGGTGTGA	SuperScript III one step RT-PCR and SequalPrep long PCR were used.	6
	7588oa	GATTACTCTGGGGAAAATTAACC		
	5482is	CAATGAAYTTTGAYCTKGAGAARTCA		
	7577ia	GGAAAATTAACCCTGATTTACTAA		

Primers for species identification					
Animal	Primer	Sequence (5' – 3')	Modifications to basic reagents, cycling conditions	Chapter	Source
Monkey	12S-L1091	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	None	5	(van der Kuyl <i>et al.</i> , 1995)
	12S-H1478	TGACTGCAGAGGGTGACGGGCGGTGTGT			
Rat	BatL5310	CCTACTCRGCCATTTTACCTATG	Annealing temperature of 48°C	6	(Herbreteau <i>et al.</i> , 2006)
	R6036R	ACTTCTGGGTGTCCAAAGAATCA			
Porcupine	CYTb1	CCATCCAACATCTCAGCATGATGAAA	Annealing temperature of 55°C	3	(Brodmann <i>et al.</i> , 2001)
	CYTb2	GCCCCTCAGAATGATATTTGTCCTCA			

Most primers were named using a combination of:

- Position (numbered relatively to K01392 for EV, NC\_001918.1 for kobuvirus, DQ401688 for cardiovirus and HM153767 for HuV)
- Orientation (os, outer sense; oa, outer antisense; is, inner sense; ia, inner antisense)

In addition, a real-time PCR protocol for porcine kobuvirus (PKV) was developed as follows. Primers and a TaqMan probe targeting a 200 bp conserved fragment in the 5'UTR of PKV were designed using guidelines from literature (Bustin & Nolan, 2013). The 200 bp fragment was first amplified from a positive sample using primers for real-time PCR and cloned into pGEM-T Easy Vector (Promega, UK). In brief, 3 µl of PCR product was mixed with 5 µl of 2X Rapid Ligation Buffer, 1 µl of pGEM-T Easy Vector (50 ng) and 1 µl of T4 DNA Ligase (3 Weiss units/µl). After incubation at 37°C for 1 hour, 2 µl of the ligation product was transformed into 50 µl of *E. coli* competent cells. The cells were recovered in SOC medium for 1 hour at 37°C before plated onto LB/ampicillin/IPTG/X-Gal plates. The resulting white colonies were screened by PCR with M13 primers using the basic protocol above and confirmed by sequencing of PCR product. A confirmed colony was grown in 10 ml of LB/ampicillin at 37°C overnight. Cells were harvested by centrifugation at 2,500 g for 5 minutes. The constructed plasmid was then isolated using QIAprep Spin Miniprep Kit (Qiagen, UK) and linearized by treatment with NsiI. The purified linear plasmid was used as template for transcription using MEGAscript T7 transcription kit (Ambion, UK). The transcription reaction (Table 2.8) was incubated at 37°C for 4 hours. DNA template was removed by incubation with 1 µl of TURBO DNase at 37°C for 15 minutes. Transcribed RNA was purified using RNeasy purification kit (Qiagen, UK). Concentration of the purified RNA was determined by Nanodrop ND1000 (Thermo Fisher Scientific, UK) and converted to copy number/µl using the website <http://www.sciencelauncher.com-MWcalc.html>. Finally, a 10-fold dilution series of RNA was prepared as mentioned above for optimization and assessment of the real-time PCR assay using SensiFAST Probe Hi-ROX kit (Bioline, UK).

**Table 2.8.** Reagents used in transcription.

Component	Amount
ATP solution	2 µl
CTP solution	2 µl
GTP solution	2 µl
UTP solution	2 µl
10X Reaction Buffer	2 µl
Linear plasmid	0.5 µg
Enzyme mix	2 µl
Nuclease free water	to 20 µl

All primers and probes for real-time PCR are presented in Table 2.7. The optimised cycling conditions of the real-time PCR assays were 1 cycle of 95°C for 5 minutes, then 45 cycles of 95°C for 15 s, and 60°C for 45 s (PKV) or 60 s (parechovirus and EV). Probes were labelled with different dyes: FAM (for EV and PKV), LCRED610 (for parechovirus) and Cy5 (for EAV control). Amplified nucleic acid was detected directly using LightCycler 480 (Roche, UK) or Rotor-Gene Q (Qiagen, UK).

**Table 2.9.** Reagents used in real-time PCR for EVs, parechovirus and PKV.

Reagent	Volume (µl)			
	EV	HPeV	LV	PKV
2x SensiFAST Probe Hi-ROX mix*	10	10	10	10
Sense primer (10 µM)	2	1	0.4	0.8
Antisense primer (10 µM)	2	1	0.4	0.8
Target picornavirus probe (10 µM)	0.4	0.2	0.2	0.4
EAV sense + antisense primer mix (10 µM)	0.4	0.4	0.4	-
EAV probe (10 µM)	0.1	0.1	0.1	-
cDNA	5	5	5	5
Nuclease free water	0.1	2.3	3.5	3

\* 2x mastermix contains all the components needed for real-time PCR, including dNTPs, MgCl<sub>2</sub>, Taq DNA polymerase, stabilizers and enhancers.

#### 2.2.3.4. SuperScript One-Step reverse transcription polymerase chain reaction (RT-PCR)

A more sensitive protocol in which both steps of cDNA synthesis and DNA amplification are performed in a single tube was used in case the optimized GoTaq protocols listed above failed to amplify the expected regions. This was obtained by using specific primers and SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen, UK). The optimized parameters and cycling conditions are as follows.

**Table 2.10.** Reagents used in one step RT-PCR

Component	Volume (µl)
2X reaction mix*	10
Outer sense primer (10 µM)	1
Outer antisense primer (10 µM)	1
SuperScript® III reverse transcriptase with platinum Taq	0.8
Extracted RNA	6
Nuclease free water	1.2

\* 2X reaction mix is supplied with the Superscript III One-step RT-PCR kit and contains 4 mM of each dNTP and 3.2 mM of MgSO<sub>4</sub>.

**Table 2.11.** Cycling conditions in one step RT-PCR

Number of cycles	Temperature	Time
1	45°C	1 hour
20	53°C	60 s
	55°C	60 s
1	70°C	15 min
1	94°C	2 min
40	94°C	30 s
	50 – 55°C*	30 s
	68°C	1 min/kb
1	68°C	5 min

\* Annealing temperatures varied among target viruses and regions to be amplified.

As primers specific for a particular region were used in the RT step and first round PCR, the resulting product could only be used as template in second round PCR (previously described) for amplification of that specific fragment. Platinum Taq DNA polymerase is complexed with a proprietary antibody that blocks polymerase activity at room temperatures. Polymerase activity is restored after the complexed antibody is denatured at high temperature of the denaturation step. This automatic “hot start” in PCR increases sensitivity, specificity, and yield (Chou *et al.*, 1992; Sharkey *et al.*, 1994).

#### 2.2.3.5. SequalPrep™ long PCR

For efficient amplification of long fragments (> 2 kb), in addition to the use of SuperScript III one-step RT-PCR in the first round PCR as described above, SequalPrep long PCR kit (Invitrogen, UK) was used in the second round PCR following the manufacturer’s instructions. Reagents and cycling conditions are detailed below.

**Table 2.12.** Reagents used in SequalPrep long PCR

Component	Volume (µl)
SequalPrep 10X reaction buffer	2
DMSO	0.4
SequalPrep 10X enhancer A	2
SequalPrep long Polymerase, 5 U/µl	0.36
Inner sense primer (10 µM)	1
Inner antisense primer (10 µM)	1
First round PCR product	1
Nuclease free water	12.24

**Table 2.13.** Cycling conditions for SequalPrep™ long PCR

Number of cycles	Temperature	Time
1	94°C	2 min
10	94°C	10 s
	50 – 55°C*	30 s
	68°C	1 min/kb
30	94°C	10 s
	52 – 56°C*	30 s
	68°C	1 min/kb (+20 s/cycle)
1	72°C	5 min

\* Annealing temperatures varied between primer sets.

### 2.2.3.6. Agarose gel electrophoresis

Products of the second round PCR were visualised by agarose gel electrophoresis. For good separation of DNA, 2% (w/v) agarose gels were used for DNA less than 1 kb in length while 1% agarose gels were used for longer DNA product. Agarose powder (Invitrogen, UK) was dissolved in 1X TAE buffer and mixed with an appropriate volume of SYBR® Safe DNA gel stain (Invitrogen, UK) recommended by the manufacturer before casting. 6 µl of product of second round PCR (using 5X green GoTaq reaction buffer) was loaded directly onto a well of prepared gel. In case 5X green GoTaq reaction buffer was not used (e.g. SequalPrep long PCR), 6 µl of PCR product was mixed well with 1.5 µl of 6X loading buffer before loaded. After electrophoresis at 150 volts for about 45 minutes, the product size was estimated by visualizing and comparing the band with a ladder electrophoresed alongside in a UV transilluminator.

### 2.2.3.7. Gel extraction and purification of amplified products

In cases additional bands were visualized on agarose gel although the PCR had been thoroughly optimised, bands of expected sizes were excised and purified using QIAquick gel extraction kit (Qiagen, UK) and the instructions of the manufacturer. In brief, 3 volumes of buffer QG were added to 1 volume of gel in a 1.5 ml tube. As the gel slice had completely dissolved after incubation at 50°C, the sample was mixed with 1 volume of isopropanol. The solution was then transferred to a QIAquick column and centrifuged at 17,900 g (13,000 rpm) for 1 minute. The column was washed with 0.75 ml of buffer PE before elution of the DNA in 50 µl of buffer EB.

#### **2.2.3.8. DNA sequencing**

Amplicons were sequenced in both senses using BigDye Terminator kit (Applied Biosystems, Warrington, UK). Each reaction contained 7 µl nuclease free water, 1 µl BigDye, 1 µl inner sense primer or antisense primer and 1 µl PCR product or purified DNA. Reactions were carried out with 25 cycles of 30 s at 96°C, 20 s at 50°C and 4 min at 60°C. Sequences were read at the Edinburgh Genomics Sequencing Service of the University of Edinburgh.

#### **2.2.3.9. Complete genome sequencing**

Additional primers were designed to obtain genomes of novel EVs and HuVs (Table 2.7). These primer sets were used to amplify regions of about 0.6 – 2.5 kb with identical overlaps between fragments. This helped prevent the possibility of hybrid viruses formed by assembly error from the same pigs or monkeys co-infected with two or more similar variants. The obtained fragment sequences from one sample were aligned and joined to produce a consensus genome sequence using SSE.

#### **2.2.3.10. Identification of animal species**

Identification of monkey species was determined by mitochondrial DNA (mtDNA) analysis as described previously (van der Kuyl *et al.*, 1995) by amplifying and sequencing a 386 bp fragment spanning the 12S gene from the extracted DNA. The sequences were then subjected to BLAST search on NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the species of monkeys.

Species identification of the other animals was carried out by colleagues at OUCRU. Species of porcupines were identified by morphology and sequencing a 359 bp fragment of the cytochrome b as previously described (Brodmann *et al.*, 2001). The obtained sequences were compared with reference sequences by BLAST search.

For rats, species identification was carried out by using protocols recommended by CERoPath (Community Ecology of Rodents and their Pathogens in South-East Asia) (Herbreteau *et al.*, 2006). Rat species were first identified on the basis of biological and morphological characteristics and confirmed by amplification using the basic



protocol above and sequencing of a conserved housekeeping gene (cytochrome oxidase subunit I, COI). The cycling conditions were similar to the basic protocol except for the annealing temperature was at 48°C. The obtained COI sequences were submitted to the RodentSEA barcoding tool ([http://www.ceropath.org/barcoding\\_tool-/rodentsea](http://www.ceropath.org/barcoding_tool-/rodentsea)), which compared the submitted sequences to a collection of reference sequences. The comparison was based on Kimura 2 Parameter distances matrix computation and phylogenetic tree reconstruction using Neighbour-Joining method (see 2.3.3).

Bats, civets and shrews were speciated based on morphology using published guides (Francis & Barrett, 2008; Kruskop, 2013).

## **2.3. Computational methods**

### **2.3.1. Statistical methods**

Statistical tests implemented in Minitab 17 (<http://www.minitab.com>) were used to compare data between groups of interest when appropriate. They included:

- Chi square test which was replaced by two-tailed Fishers exact tests if any of the cells of a contingency table were below 5
- Kruskal-Wallis non-parametric test
- Mann-Whitney test

### **2.3.2. Sequence alignment**

Sequence alignment is important and essential for downstream phylogenetic and evolutionary analyses. Correct alignment helps to avoid false conclusions on phylogeny, genetic diversity and recombination. PCR products were sequenced in both directions to allow for dual coverage. Sequences were first imported into DnaBaser ([www.DnaBaser.com](http://www.DnaBaser.com)) for assembly. By comparing the quality of signal peaks between sense and antisense strands from a sample, DnaBaser could automatically identify and resolve mismatches (if any) between the two sequences. Mismatches were also highlighted for easy confirmation by visual inspection of chromatograms. Assembled sequences were then imported into an SSE version 1.1 (Simmonds, 2012)

file which also included at least 1 reference sequence previously downloaded from GenBank for each of all (sero)types from a species or genus under study. The sequences from studied samples were subjected to initial alignment to the best sequence match and subsequent global alignment using Muscle version 3.8 (Edgar, 2004) implemented within SSE. The global alignment could be at the level of nucleotides (for non-coding regions) or codons (for coding regions) which were obtained by translation of nucleotide sequences using the standard genetic code available in SSE. Alignments were refined where necessary by manual editing.

### 2.3.3. Construction of phylogenetic trees

A phylogenetic tree is a graph that depicts the evolutionary relationships between organisms or gene sequences. Its construction has been a standard part of exploratory sequence analysis (Holder & Lewis, 2003). Phylogenetic trees can be constructed by a variety of methods, some of which are briefly summarized in Table 2.14.

**Table 2.14.** Summary of methods commonly used for phylogenetic tree construction. Adapted from (Holder & Lewis, 2003).

Method	Advantages	Disadvantages
Neighbour joining	Fast	<ul style="list-style-type: none"> <li>- Loss of information when sequences are converted into distances</li> <li>- Hard to obtain reliable estimates of pairwise distances for divergent sequences</li> <li>- Exposed to LBA*</li> </ul>
Parsimony	<ul style="list-style-type: none"> <li>- Fast</li> <li>- Robust for short branches (closely related sequences/ dense sampling)</li> </ul>	<ul style="list-style-type: none"> <li>- Can perform poorly in case of substantial variation in branch lengths</li> <li>- Exposed to LBA</li> </ul>
Minimum evolution	Uses models to correct for unseen events (e.g. back mutations)	Corrections can break down for large distances
Maximum likelihood	Fully captures what data tell us about phylogeny under a given model	Computationally intensive and therefore slow
Bayesian	Faster than ML bootstrapping in assessing support for trees and provides measures of uncertainty	<ul style="list-style-type: none"> <li>- The prior must be specified</li> <li>- Difficult to determine whether the Markov chain Monte Carlo approximation has run for long enough</li> </ul>

\* LBA, long-branch attraction, the tendency of distantly related sequences to group closely in a tree regardless of their true relationships (Baldauf, 2003).

The use of neighbour-joining (NJ) is extremely popular (Gascuel & Steel, 2006; Holder & Lewis, 2003). This distance based method, like others, performs well on low divergent sequence data. Furthermore, NJ is fast as it is more computationally efficient than maximum parsimony based methods. This feature enables analysis of large datasets from a number of operational taxonomic units. However, it can be difficult to achieve reliable distance matrix values for distantly related input sequences (Holder & Lewis, 2003).

From many perspectives, maximum likelihood (ML) is the most appealing method for phylogeny estimation. The likelihood function has proved to be consistent and powerful for statistical inference. ML applies evolutionary models that incorporate parameters to govern aspects of the process of evolution, such as the degree to which the rate of evolution differs across sites. All possible mutational pathways, including unseen events (such as back mutations or complex pathways like A→C→G along the branch) compatible with the data are taken into account. As ML corrects for multiple mutational events at the same site, it accurately reconstructs the relationships between sequences that have been separated for a long time, or are evolving rapidly (Holder & Lewis, 2003). The phylogeny with the highest overall likelihood score is selected after calculations of the probability of the topology, branch lengths and substitution model parameters (Fukami-Kobayashi & Tateno, 1991). However, the main obstacle that prevents the widespread use of ML is the computational burden as the algorithm must search through a multidimensional space of parameters to find the ML score (Holder & Lewis, 2003; Rogers & Swofford, 1998).

In consideration of advantages and disadvantages of these methods, ML was used in this thesis. Unless otherwise stated, phylogenetic trees used for identification of virus types and recombination analyses were reconstructed using ML method implemented in the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.05 (Tamura *et al.*, 2011) with 1000 bootstrap replications (see section 2.3.4) and partial deletion for missing data. The best substitution models (those with the lowest Bayesian Information Criterion scores) were determined for each data set using MEGA prior to tree construction. Trees were rooted by inclusion of an outlier (e.g. a sequence belonging to a closely related species / genus) specified under each tree. Rooting of a

tree provided an estimate of the ultimate common ancestor of all the studied viruses (Hall, 2013).

#### **2.3.4. Bootstrapping**

The bootstrap is a statistical technique that is used to determine the robustness of observed phylogenetic groupings within a tree. Sequence data matrix is first randomly re-sampled with replacement and trees are then produced from each of the resulting pseudo-replicate data sets. The proportion of times that a group of interest is obtained in the pseudo-replicates is the bootstrap support value for that group (Alfaro *et al.*, 2003). Bootstrapping therefore offers an estimate of the reproducibility of a monophyletic clade or helps predict whether the same result would be observed if more data were collected, not whether the result is correct (Holder & Lewis, 2003). Bootstrap proportions are conservative measures of support, so values above the threshold of 70% are considered as strong support for a group (Efron *et al.*, 1996; Hillis & Bull, 1993; Zharkikh & Li, 1992). In contrast, a grouping with a low bootstrap support value implies that if more data were collected, it is highly possible that the group would not be reproduced (Holder & Lewis, 2003).

One major limitation of bootstrapping is that it merely indicates how robust the tree phylogeny is and fails to provide an estimate of the probability of an observed tree. For example, in case a tree is obtained with long-branch attraction, bootstrapping might indicate relatively strong support regardless of their true relationships (Swofford *et al.*, 2001). Another limitation of bootstrapping, like ML, is the computational burden (Holder & Lewis, 2003).

#### **2.3.5. Recombination detection methods**

A number of methods have been developed for detection of recombination in a set of aligned sequences. They can be classified into four categories (Posada & Crandall, 2001) of distance methods, compatibility methods, substitution distribution methods and phylogenetic methods. Evaluation on the performance of 14 different recombination detection methods using empirical data sets (Posada, 2002) showed they often give differing or even, contradictory results. For this reason, the author

suggested definitive conclusions about the occurrence of recombination should not be based on a single method.

Phylogenetic methods are the most commonly used methods in the literature. These methods infer recombination when phylogenies from different genome regions are incongruent (Posada, 2002). They were also the basis of recombination analysis in this work. Phylogenetic trees constructed from different genome fragments of varying sizes were examined for phylogeny violations between bootstrap supported ( $\geq 70\%$ ) clades. In order to verify putative events and determine recombination breakpoints, all detected violations were further studied by analysis with sequence divergence plot and Group Scan (Simmonds & Midgley, 2005) available in the SSE package. The former (Simmonds, 2012) uses sliding window with pre-specified fragment length and increment to calculate pairwise distances between a query group and control groups. Sequence distances are plotted on the y axis of an output graph with genome position on the x axis. An abrupt decline into intra-type or intra-species divergence levels indicates potential recombination. The latter measures the extent a query sequence is embedded within each clade formed by standard phylogenetic analysis of preassigned groups. Grouping scores range from 0 (no grouping) to 1 (deeply clustered within a clade). To identify potential recombination sites, successive fragments with predefined length and increment between fragments are analyzed (in this thesis, a fragment size of 300 nucleotides and an increment of 30 were used). An output graph is plotted as midpoints of the analysed fragments on the x axis and grouping scores on the y axis. A putative breakpoint is determined by point of intersection where abrupt changes in grouping values of the sequence occur.

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## Chapter 3. Prevalence of picornaviruses in screened samples

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### 3.1. Introduction

Numerous logistical and scientific challenges exist in documenting emerging zoonotic infections (Keusch *et al.*, 2009). Monitoring is subject to massive ascertainment biases (e.g. differences in the efforts invested in different places and at different times). It is performed in an *ad hoc* manner, partly driven by responses to the most recent events (e.g. discovery of coronaviruses following the SARS outbreak) and by availability of detection and identification technologies. New species pathogenic to humans continue to be discovered and determining the number of pathogens that conceivably exist in mammalian and other reservoirs is difficult (Woolhouse & Gaunt, 2007). The frequency with which humans are exposed to animal pathogens, and the significance of those exposure events therefore remains to be clearly determined. Finally, the nature of the species barrier and the factors that allow pathogens to cross it and establish transmission between humans are critical issues that need to be addressed (Taylor *et al.*, 2001).

All the challenges mentioned above lead to many gaps in current knowledge of emerging pathogens. This also holds true for picornaviruses. Six picornavirus genera including *Enterovirus*, *Parechovirus*, *Cardiovirus*, *Kobuvirus*, *Cosavirus* and *Hunnivirus* were of interest and selected for studies in this thesis as they comprise important human and animal pathogens (e.g. HFMD virus, FMD virus), viruses with growing evidence for zoonotic potential (EVs, HPeVs) or new members with unknown ability to cause disease (EVs, cosaviruses, cardioviruses, HuVs). The list of picornaviruses with zoonotic potential is expanding with the number of studies conducted. The detection of a significant number of (sero)types of human EV in a rabbit (O'Connor & Morris, 1955), a fox (Makower & Skurska, 1957), a bat and rats (Gregorio *et al.*, 1972), pigs (Grew *et al.*, 1970; Lomakina *et al.*, 2015), dogs and chickens (Graves & Oppenheimer, 1975), non-human primates (Harvala *et al.*, 2011b; Oberste *et al.*, 2013a; Oberste *et al.*, 2013b; Sadeuh-Mba *et al.*, 2014) and HPeV (Oberste *et al.*, 2013b; Shan *et al.*, 2010) is evidences for cross-species transmission;

non-human primates can conversely be a source of picornavirus infections in humans. However, whether other picornaviruses, particularly the newly discovered EV types, cosavirus, cardiovirus and HuV also have zoonotic potential and disease association, and whether other animals carry zoonotic picornaviruses is unknown. Information has been reported separately from either humans or animals and from different countries.

In Vietnam, some of the targeted picornavirus genera have been described only from one side (normally humans) of the human-animal interface. The existence (if any) of these viruses in non-human hosts and the relationships between viruses in animals and humans have never been studied. Among the six picornavirus genera, only *Enterovirus* has been frequently documented from patients with HFMD (Geoghegan *et al.*, 2015; Khanh *et al.*, 2012), encephalitis or meningitis (Takamatsu *et al.*, 2013; Tan *et al.*, 2010; Tan *et al.*, 2014; Taylor *et al.*, 2012) or diarrhoea (Phan *et al.*, 2005). The presence of *Kobuvirus* in river water (Inaba *et al.*, 2014) and human samples (Pham *et al.*, 2007), *Cardiovirus* in rats (Buckwalter *et al.*, 2011) and absence of *Parechovirus* (Tan *et al.*, 2010) in clinical samples have occasionally been reported, while no studies on *Hunnivirus* and *Cosavirus* have been conducted.

Meanwhile, it is known that the emergence of zoonotic pathogens in humans depends on interactions between humans and the animal reservoir and/or vectors or their environment (Gortazar *et al.*, 2014). VIZIONS is responding to the need for active monitoring and research programmes on human and animal pathogen reservoirs by establishing a model for integrated human and animal surveillance. In the first step to address these gaps, the study in this chapter aimed to screen faecal samples collected in Vietnam and monkeys in Cameroon for the 6 picornavirus genera. Viruses in positive samples are further characterized and described in subsequent chapters.

### **3.2. Samples**

As zoonoses can originate from domestic or wild animals (Christou, 2011), samples from both these categories were included. More than 1,900 faecal samples from high risk human populations and animals collected in Vietnam and over 100 faecal samples from monkeys in Cameroon were used in this study (Table 2.1, Chapter 2).

### **3.3. Methods**

RNA was extracted from 10% (w/v) faecal suspensions and used for cDNA synthesis. Screening of the synthesized cDNA for the existence of picornaviruses was performed using nested PCR which amplified the 5'UTR (EV in monkeys, cardiovirus, cosavirus and HuV) or 3D (kobuvirus), and real-time PCR targeting the 5'UTR (enterovirus in other samples, parechovirus). Samples were screened in pools of 5 or individually depending on the initial prevalence in the first 50 randomly selected samples. Individual samples from positive pools were subsequently screened using the same methods (Chapter 2). Screening of EV in domestic pigs was completed by Ms. Pham Hong Anh at OUCRU.

### **3.4. Results**

Viruses present in the screened samples included kobuvirus, EV, cardiovirus and HuV (Table 3.1). Among these, kobuvirus was the most common virus, detected in a range of hosts (pigs, farm-bred boars, rats, porcupines, civets and humans) with detection frequencies from 0.4% in humans to 42.2% in farm-bred boars. It was followed by EV, observed at low frequencies in humans (3.2%), farm-bred boars (8.9%), and at extremely high frequencies in domestic pigs (79%), monkeys (96.5%). Cardiovirus was most commonly detected in rats and porcupines (6.6% and 4.9%, respectively), rarely found in pigs and one human sample. Among the screened samples, HuV was only evident in rats at relatively high overall frequency (32.5%). Parechovirus and cosavirus were absent in all screened samples. All duck boot swabs, chicken boot swabs, and samples from shrews and all 30 species of bats were negative for the targeted picornaviruses. Therefore, no individual chicken and duck samples were considered further for this study.



**Table 3.1.** Detection of picornavirus RNA in screened samples.

Animal	Tested	Positive (%)					
		EV	Kobuvirus	Cardiovirus	Cosavirus	Parechovirus	HuV
<b>Pig</b> ( <i>Sus scrofa</i> )	<b>682</b>	<b>539 (79.0)</b>	<b>200 (29.3)</b>	<b>1 (0.15)</b>	0	0	Not done
<b>Farm-bred boar</b> ( <i>Sus scrofa</i> )	<b>45</b>	<b>4 (8.9)</b>	<b>19 (42.2)</b>	0	0	0	0/45
<b>Rat</b> (7 species) <sup>b</sup>	407	0	<b>103 (25.3)</b>	<b>27 (6.6)</b>	0	0	<b>54 (32.5)</b> <sup>a</sup>
<b>Shrew</b> ( <i>Suncus murinus</i> )	60	0	0	0	0	0	0
<b>Porcupine</b> ( <i>Hystrix brachyura</i> )	61	0	<b>1 (1.6)</b>	<b>3 (4.9)</b>	0	0	0
<b>Civet</b> ( <i>Paradoxurus hermaphroditus</i> )	30	0	<b>5 (16.7)</b>	0	0	0	0
<b>Bat</b> (30 species) <sup>c</sup>	158	0	0	0	0	0	Not done
<b>Duck</b> (boot swab)	120	0	0	0	0	0	Not done
<b>Chicken</b> (boot swab)	120	0	0	0	0	0	Not done
<b>Monkey</b> (3 species) <sup>d</sup>	113	<b>109 (96.5)</b>	Not done	Not done	Not done	Not done	Not done
<b>Human</b> <sup>e</sup>	<b>250</b>	<b>8 (3.2)</b>	<b>1 (0.4)</b>	<b>1 (0.4)</b>	0	0	0

<sup>a</sup> Only 166 rats were screened for HuV.<sup>b</sup> Details of rat species are presented in Figure 6.3, Chapter 6.<sup>c</sup> Details of bat species are presented in Table 2.2, Chapter 2.<sup>d</sup> Details of monkey species are available in Table 1, Chapter 5.<sup>e</sup> Details of positive human samples are available in Table 7.1, Chapter 7.

### 3.5. Discussion

This is the first large scale study on the prevalence of the six picornavirus genera in adult humans (aged 21 – 64 years old) and a wide range of other animal hosts. The prevalences of these viruses were different between hosts.

#### 3.5.1. EV

Many studies have shown the existence of EVs in pigs, boars, monkeys and humans. The overall detection frequency of EVs in domestic pigs (79%) was notably higher than those reported in Spain (0%), Italy (7.5%), China (8.3%), Hungary (13.3%) and the Czech Republic (50.2%) (Boros *et al.*, 2011b; Buitrago *et al.*, 2010; Pročkalová, 2012; Sozzi *et al.*, 2010; Yang *et al.*, 2013). In contrast, detection of EV RNA in farm-bred boars in this study was much lower than prevalences of 50% in Hungary (Boros *et al.*, 2012a) and 69.4% in the Czech Republic (Pročkalová, 2012). Differences in primer set usage, pig density and farming practices between countries may have resulted in a wide range of reported EV prevalence.

In comparison with detection frequency (96.5%) of EV in non-human primates in the current work, other studies have shown lower frequencies of 5.4% (Sadeuh-Mba *et al.*, 2014) and 7.4% (Harvala *et al.*, 2011b) in Cameroon, 10% (Harvala *et al.*, 2014) in Cameroon and Democratic Republic of Congo, 8.8% (Oberste *et al.*, 2013b) and 60.2% (Oberste *et al.*, 2013a) in Bangladesh, 66% (Nix *et al.*, 2008) in the US.

Finally, EV has been frequently documented from human adults, mostly from respiratory samples (Billaud *et al.*, 2003; Lu *et al.*, 2014; Parody *et al.*, 2007; Tran *et al.*, 2012; Xiang *et al.*, 2012). Detection of EV in faecal samples from human adults has been reported only in several studies at high prevalence (96%) in patients with meningitis in Finland (Kupila *et al.*, 2005), and in 1 of 3 HFMD patients in China (Li *et al.*, 2013). In contrast, EV was less common in the high risk cohort human populations (prevalence of 3.2%) in this study who developed different symptoms (fever, pharyngitis, flu-like, etc.) at the time of sampling. This may be the result of differences in clinical status and geography of studied subjects.

Samples from the other animals were negative for EV, which has never or rarely been documented from those animals.

### 3.5.2. Kobuvirus

Studies have indicated that PKV is endemic in pigs and distributed worldwide. The PKV detection frequency (29.3%) in pigs in the current study was lower than previously reported in most other countries ranging from 31.1 – 99% (Barry *et al.*, 2011; Chen *et al.*, 2013; Khamrin *et al.*, 2010; Khamrin *et al.*, 2009; Okitsu *et al.*, 2012; Park *et al.*, 2010; Reuter *et al.*, 2010b). However, a lower rate of 13.1% was reported in Japan (Amimo *et al.*, 2014). In boars, PKV has only been documented in 10 of 10 samples in Hungary (Reuter *et al.*, 2013) while the virus was present in 42.2% of VIZIONS samples.

Very little is known about kobuvirus infection frequencies in rats. Only one study detected this virus at significantly higher frequency (50%) in rat faecal samples (Firth *et al.*, 2014) than that in the current study (25.3%). No kobuvirus has been previously reported from porcupines and civets. Therefore, this is the first study showing the existence of kobuvirus in these animals at frequencies of 1.6% (95% confidence interval [CI] 0 – 4.75%) and 16.7% (95% CI 3.35 – 30%), respectively.

For humans, most reports of kobuvirus were from children (Chang *et al.*, 2013; Kaikkonen *et al.*, 2010; Pham *et al.*, 2007; Reuter *et al.*, 2009; Sdiri-Loulizi *et al.*, 2008; Yang *et al.*, 2009). Several studies of faecal samples from adults with gastroenteritis detected AiV-1 at low frequencies (0.1 – 2%) in Canada (Houde *et al.*, 2010), Thailand (Saikruang *et al.*, 2014), France (Ambert-Balay *et al.*, 2008) and Germany (Drexler *et al.*, 2011). Exceptionally, the highest prevalence of kobuvirus reported up to date is 20.1% in gastroenteritis outbreaks associated with oysters in Japan (Yamashita *et al.*, 2000). The detection frequency of 0.4% in this chapter, therefore, falls within the range described in most previous studies.

### 3.5.3. Cardiovirus

Cardioviruses have been frequently detected from pigs in association with reproductive failure or myocardial lesions (Castrick *et al.*, 1996; Koenen *et al.*, 1991; Koenen *et al.*, 1996; Love & Grewal, 1986) by viral isolation rather than molecular

methods. Similarly cardioviruses have been isolated from rats (Drake *et al.*, 2008; Hemelt *et al.*, 1974; Ohsawa *et al.*, 2003). Only one study (Firth *et al.*, 2014) reported thera virus and Boone cardiovirus in 14% and 28% of rat faecal samples, respectively, by molecular methods. No studies have been performed to detect these viruses in porcupines while SAFV has been shown absent in adult faecal samples (Chiu *et al.*, 2008; Drexler *et al.*, 2008b; Tsukagoshi *et al.*, 2011). This study showed that in Vietnam, infection with cardioviruses was rare in pigs (detection frequency of 0.15%), humans (0.4%), and less prevalent in rats (6.6%) than previously reported. The current study additionally demonstrated the existence of the viruses in porcupines (4.9%, 95% CI 0 – 5.42%).

#### **3.5.4. Parechovirus**

HPeVs of the *Parechovirus A* species have been detected in monkeys in China (Shan *et al.*, 2010) and Bangladesh (Oberste *et al.*, 2013b) at frequencies of 5.2% and 3.1%, respectively. HPeVs have also been documented from children at low frequencies (Benschop *et al.*, 2008b; Ghazi *et al.*, 2012; Harvala *et al.*, 2011a) and in faecal samples from 9/14 Japanese adult patients with myalgia (Mizuta *et al.*, 2013). This suggested that infection with HPeV is not limited to the pediatric population. However, none of the screened samples were positive for HPeV. The absence of HPeV in human adult samples is consistent with a previous study using samples from Vietnamese children (Tan *et al.*, 2010). Overall, HPeV infection is not common in Vietnam.

*Parechovirus B* has been reported from bank voles (Hauffe *et al.*, 2010; Lindberg & Johansson, 2002; Niklasson *et al.*, 1999), mice (Johansson *et al.*, 2003) and rats only, with a prevalence of 24.4% in voles and mice in the UK (Salisbury *et al.*, 2014). This study used the same primer set with the study in the UK. Therefore the absence of *Parechovirus B* likely reflected differences not in the assay but in the prevalence of the virus between vole, mouse and rat species. *Parechovirus B* was absent in all screened samples, including 275 rats purchased and trapped in 5 provinces of the Mekong delta. This is in accordance with finding in a seroprevalence study (Nguyen *et al.*, 2015) which showed none of the sera from those rats were seroreactive to LV.

### **3.5.5. Cosavirus**

No cosavirus RNA was detected in any screened animal samples, consistent with the fact that this virus has been reported from humans only (primarily from children). No human samples (all from adults) were positive for cosavirus either although this virus has been demonstrated elsewhere from faecal samples. A study in Thailand (Khamrin *et al.*, 2012) detected HCoV RNA in 1 of 150 samples from adults with diarrhea. Another study in Nepal (Kapusinszky *et al.*, 2012) showed HCoV RNA prevalences of 12% and 15% in adults with diarrhea and healthy adults, respectively. Finally, persistent infection with HCoV was associated with chronic diarrhea in an adult double lung transplant recipient (Campanini *et al.*, 2013).

### **3.5.6. Hunnivirus**

Although the first HuV was isolated in 1969 (McFerran *et al.*, 1969), it was only recently recognized as a member of a new genus *Hunnivirus*. As HuVs have been detected in some animals such as cattle and sheep in Hungary and Northern Ireland (Reuter *et al.*, 2012) with unknown pathogenicity and zoonotic potential, not much attention has been paid to this virus genus. Recently a single study detected HuV RNA from 19% of rat faecal samples in the US (Firth *et al.*, 2014) – a lower prevalence than in the current study. No samples from other screened animals and humans were positive for this virus.

### **3.5.7. Absence of the screened picornaviruses in bats, chickens and ducks**

Bats, chickens and ducks were included in this study as they are well documented as sources of many important zoonotic pathogens such as Middle East respiratory syndrome coronavirus (Ziad *et al.*, 2013), Ebola (Leroy *et al.*, 2005), avian influenza (Beigel *et al.*, 2005). However, picornaviruses remain a neglected group in studies of bats (Kemenesi *et al.*, 2015) as well as chickens and ducks. As a result, among the targeted picornaviruses, only CVB4 (Graves & Oppenheimer, 1975), CVB3 (Gregorio *et al.*, 1972) and kobu-like viruses (Li *et al.*, 2010) have been identified from bats and chickens. This may also indicate that infection with viruses of the 6 picornavirus genera in these animals is rare. Therefore, it may be not surprising that none of the screened picornaviruses were detected in bats, chickens and ducks.

In conclusion, in common with previously published data, the current study showed that the infection frequencies of the targeted picornaviruses varied quite considerably with hosts. In addition to detection of picornaviruses in hosts previously known to carry the viruses, this study demonstrated evidence for their existence in other animals (kobuviruses in porcupines and civets, cardioviruses in porcupines). Whether they were associated with age of infected hosts, disease (where clinical information was available) and whether there was any overlap between viruses circulating in animals and humans will be elucidated in subsequent chapters.

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## Chapter 4. Enterovirus and kobuvirus infection in domestic pigs and boars

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### Introduction

In the last chapter, 682 faecal samples from domestic pigs and 45 from boars were screened for picornaviruses. The screening results showed that infection with EV was endemic in domestic pigs (detection frequency of 79%) and less common in boars (8.9%). In contrast, detection frequency of kobuvirus in boars (42.2%) was substantially higher than that in domestic pigs (29.3%).

In this chapter, I will examine the molecular epidemiology and disease associations of these viruses. The genetic diversity of EVs and kobuviruses in pigs and boars, and recombination of EV will also be studied. Findings from this chapter will additionally help understand if these animals in Vietnam are infected with any EVs or kobuviruses that can also infect humans. Together, the findings will elucidate the hypothesis of disease association, incomplete genetic characterization and zoonotic potential of these viruses.

### Pathogenicity of EVs and kobuviruses infecting pigs and boars

Before the studies described in this chapter were performed, EVs and kobuviruses infecting pigs and boars comprised 5 types in the species *Enterovirus G* (which also consists of 2 ovine EV types), CVA20 (Grew *et al.*, 1970), CVB4 (Lomakina *et al.*, 2015), swine vesicular disease virus (SVDV) in *Enterovirus B* and PKV-1 in the species *Aichivirus C* (<http://www.picornaviridae.com>, accessed October 15, 2013). Among these, only SVDV is known to be pathogenic, causing an acute, contagious swine vesicular disease characterized by the development of vesicles and erosions on the legs and around the mouth (Spickler *et al.*, 2010). Since first described in Italy in 1966 (Nardelli *et al.*, 1968), outbreaks of SVDV have been documented throughout Europe and Asia (Jimenez-Clavero *et al.*, 2005). SVDV is antigenically related to human virus coxsackievirus B5 (CVB5) and is classified as a porcine variant of CVB5 (Brown *et al.*, 1973; Knowles & McCauley, 1997; Seechurn *et al.*, 1990).

Seroconversion to SVDV in laboratory workers handling the agent has been reported. However, no farmers or veterinarians working with infected pigs have been reported with seroconversion or disease. Experimental infections have also failed to show transmission of CVB5 between pigs (Spickler *et al.*, 2010). In contrast, little is known about possible pathogenicity of EV-G and Aichivirus C. EV-G was first isolated from skin lesions of pigs (Knowles, 1988). Its ability to cause disease has only been indicated in one experimental infection study (Yang *et al.*, 2013) which showed that two of the twelve pigs infected with EV-G1 developed flaccid paralysis of the hind limbs. Similarly, Aichivirus C has only been reported in association with diarrhoea in pigs (Chen *et al.*, 2013; Park *et al.*, 2010).

### **Definition of new EV types based on sequence divergence in the VP1 region**

Sequence based methods have been developed to overcome disadvantages (time-consuming, labor-intensive, costly and limited supply of antisera) of the traditional serotyping by virus isolation and neutralization tests. In those methods, different genome regions (VP4, VP2, VP1 and 3D) were targeted. Among these, VP1 encodes for the most external and immunodominant protein of the picornavirus capsid (Rossmann *et al.*, 1985) and recombination has rarely been described in this region (Blomqvist *et al.*, 2003; Zhang *et al.*, 2010). As a result, the best region for molecular identification of EV type was VP1 (Casas *et al.*, 2001; Perera, 2010) whose sequences consistently correlate with serotype designations of EVs (Kiang *et al.*, 2009; Kilpatrick *et al.*, 1998; Oberste *et al.*, 1999). For EV-A – EV-D, members of the same serotypes show less than 25% nucleotide or 12% amino acid sequence divergence while between serotype distances are consistently greater than these thresholds (Oberste *et al.*, 1999). These thresholds have been adopted by the ICTV Picornavirus Study Group as an alternative to neutralization assays for the identification of EVs and assignments of new EV types. This genotypic approach has advantages over serotyping of accuracy, speed and ability to identify and assign new types (Oberste *et al.*, 2000; Kiang *et al.*, 2009). A large number of new EV types have been identified on this basis (Brown *et al.*, 2009; Harvala *et al.*, 2011b; Harvala *et al.*, 2014; Oberste *et al.*, 2007; Oberste *et al.*, 2002; Oberste *et al.*, 2004b; Sadeuh-Mba *et al.*, 2014; Smura *et al.*, 2007).



This chapter is comprised of studies published in two papers. The pilot study (Nguyen *et al.*, 2014a) examined EVs in 198 samples from pigs only while the larger scale study (Nguyen *et al.*, 2015) focused on both EVs in the rest 484 pig, 45 boar samples and kobuviruses in all 682 pig and 45 boar samples.

**Prevalence, genetic diversity and recombination of species G  
enteroviruses infecting pigs in Vietnam**

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## **Prevalence, genetic diversity and recombination of species G enteroviruses infecting pigs in Vietnam**

Picornaviruses infecting pigs, described for many years as “porcine enteroviruses”, have recently been recognized as distinct viruses within three distinct genera (*Teschovirus*, *Sapelovirus* and *Enterovirus*). To better characterize the epidemiology and genetic diversity of members of the *Enterovirus* genus, faecal samples from pigs from four provinces in Vietnam were screened by PCR using conserved enterovirus (EV)-specific primers from the 5' untranslated region (5'UTR). High rates of infection were recorded in pigs on all farms, with detection frequencies of approximately 90% in recently weaned pigs but declining to 40% in those aged over 1 year. No differences in EV detection rates were observed between pigs with and without diarrhoea [74% (n = 70) compared with 72% (n = 128)]. Genetic analysis of consensus VP4/VP2 and VP1 sequences amplified from a subset of EV-infected pigs identified species G EVs in all samples. Among these, VP1 sequence comparisons identified six type 1 and seven type 6 variants, while four further VP1 sequences failed to group with any previously identified EV-G types. These have now been formally assigned as EV-G types 8 – 11 by the Picornavirus Study Group. Comparison of VP1, VP4/VP2, 3Dpol and 5'UTRs of study samples and those available on public databases showed frequent, bootstrap-supported differences in their phylogenies indicative of extensive within-species recombination between genome regions. In summary, we identified extremely high frequencies of infection with EV-G in pigs in Vietnam, substantial genetic diversity and recombination within the species, and evidence for a much larger number of circulating EV-G types than currently described.

### **INTRODUCTION**

A wide variety of viruses infect the domestic pig (*Sus scrofa*) and many, such as rotaviruses and caliciviruses, are associated with severe enteric disease. A large group of viruses, collectively termed “porcine enteroviruses”, members of the virus family *Picornaviridae*, have also been implicated in a wide range of disease presentations in pigs (Knowles, 2006; Knowles *et al.*, 1979). A better understanding of their

epidemiology and pathogenicity was achieved once it was recognized that the group incorporated members of three different picornavirus genera, *Teschovirus*, *Sapelovirus* and *Enterovirus*, with distinct infection profiles and disease associations (Kaku *et al.*, 2001; Knowles, 2006; Krumbholz *et al.*, 2002). Among these, teschoviruses have been identified as the cause of a frequently severe epidemic form of encephalomyelitis, as well as a range of other systemic disease manifestations including diarrhoea, respiratory disease and myocarditis. Sapeloviruses have also been linked with enteric and respiratory disease presentations and more recently, with polioencephalomyelitis (Lan *et al.*, 2011).

The third group, now classified as members of the genus *Enterovirus*, were originally isolated from skin lesions of pigs (Knowles 1988) but have not to date been clearly linked to enteric or other disease presentations (Knowles 2006). Enteroviruses (EVs) infecting pigs are genetically distinct from other EVs and have recently been assigned as members of species G (EV-G) (Knowles *et al.*, 2012), a separate species from those infecting humans (species A – D), cows (E and F) and non-human primates (A, B, D, H and J). The original isolates of EV-G (PEV-9 and PEV-10) were serologically distinct from each other and are now recognized and reassigned as separate EV-G types, EV-G1 and -G2. Very recently, molecular methods have identified further genetic variants, now classified as EV-G3 and EV-G4 from pigs and wild boars in Hungary (Boros *et al.*, 2012a; Boros *et al.*, 2011), EV-G5 from a sheep (Boros *et al.*, 2012b) and EV-G6 from a pig in Korea (Moon *et al.*, 2012). These type assignments were based on sequence divergence of the VP1 gene, where types display 25% nucleotide sequence divergence from each other (<http://www.picornaviridae.com/>); these are a substitute for demonstrating serological relationships, which remain as yet uncharacterized for these newly assigned types.

In the current study, we investigated the infection frequency, disease associations and genetic diversity of EVs in pigs from Vietnam. This study was part of a larger longitudinal cohort research programme within farming communities in Vietnam, providing a baseline survey of enteric pathogens on pig farms in the study province, Dong Thap, located in the Mekong delta.

## RESULTS

### Infection frequency of EV

Detection frequencies of EV RNA were high within all four surveyed districts of Dong Thap province, with 92 of 102 (90%) farms testing EV positive for at least one pig. Rates of infection were highest among weaners (93%) aged 7 – 14 weeks, and declined moderately with the age of the pigs, with 42% of those older than 1 year of age continuing to shed detectable levels of EV RNA (Table 1). Frequencies of EV detection in pigs with and without diarrhoea were similar in all age ranges. There was no significant association between viral load as determined by EV threshold cycle (Ct) values and clinical status (data not shown).

**Table 1.** Detection of EV RNA in pig faecal samples

Group	Age range (weeks)*	With diarrhoea		Without diarrhoea		<i>p</i> -value**
		Pigs tested (N)	EV frequency (%)	Pigs tested (N)	EV frequency (%)	
Suckler	< 7	9	89	33	85	1.0
Weaner	7 – 14	29	97	29	90	0.66
Grower	15 – 23	10	70	28	71	1.0
Gilts	24 – 52	0	-	12	58	-
Sows, boars	> 52	22	41	26	42	1.0
Total	-	70	74	128	72	0.74

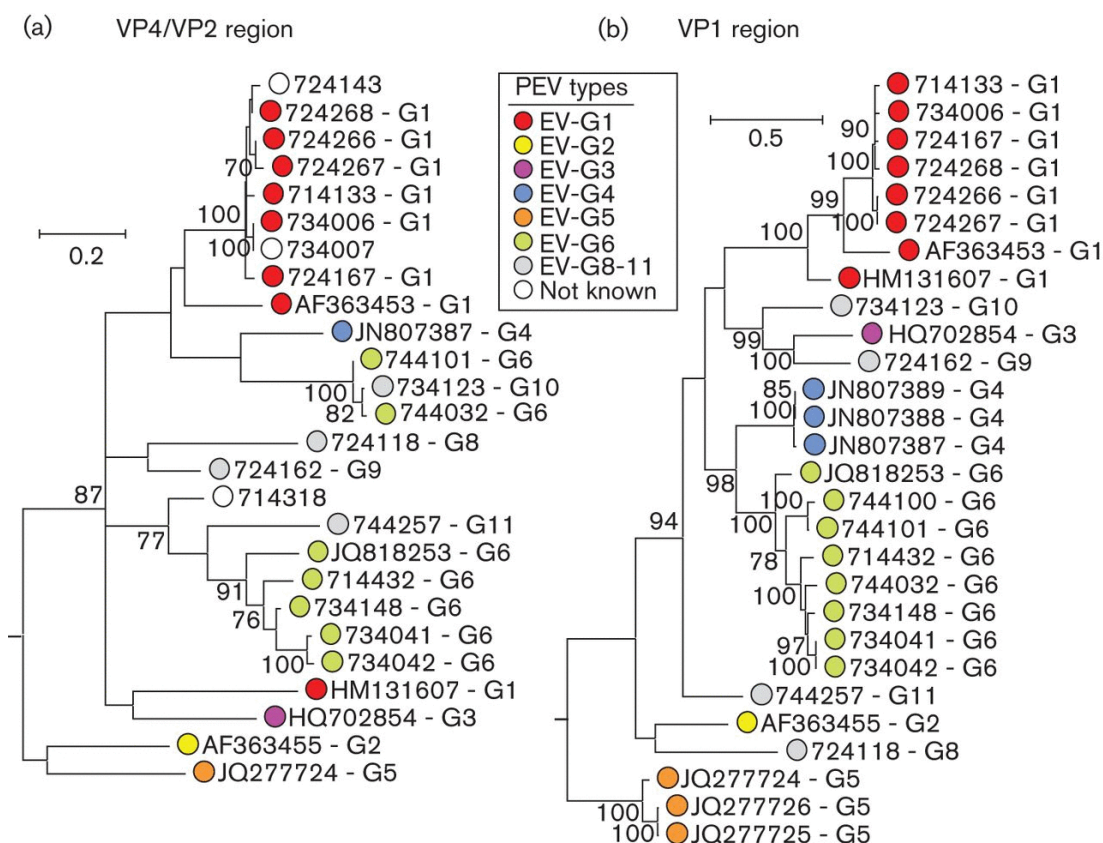
\* Typical age range for group.

\*\* Two-tailed Fisher's exact test.

### Genetic diversity of EV variants

The genetic diversity of EVs detected in a subsample of 20 positive faecal specimens was determined by sequence comparisons in the VP4/VP2 and VP1 regions (Figure 1). All but one sample could be amplified in the VP4/VP2 region, while 17 of 20 were positive in VP1. Consensus sequences from these two regions identified species G EV variants in all infected pigs. The latter region was additionally used for type assignments; phylogenetic analysis of this region identified six type 1 (EV-G1) variants, seven EV-G6 and four further variants that did not cluster with sequences from this study or with those previously published and available in public sequence databases. These latter variants showed > 25% nucleotide sequence divergence from other EV-G types over the VP1 coding sequences and on consultation with the ICTV

Picornavirus Study group, they have been assigned as new types EV-G8 – EV-G11 (<http://www.picornaviridae.com/enterovirus/ev-g/ev-g.htm>).



**Figure 1.** Phylogenetic comparisons of sequences from VP4/VP2 region (nt 810 – 1250) (a) and VP1 (nt 2469 – 3317) (b) from study samples and available sequences of other EV-G variants from GenBank. Maximum-likelihood trees were reconstructed using 500 bootstrap resamples to demonstrate the robustness of groupings; values of  $\geq 70\%$  are shown. The tree was rooted by inclusion of the more divergent EV (bovine) species E variant, GenBank accession no. AF123432 (not shown). The tree was drawn to scale; bar shows indicated evolutionary distance.

Pigs infected with type 1 were typically younger (median age 4 weeks, range 3 – 9) than those infected with type 6 (median 7 weeks, range 5 – 56) and other types (median 104 weeks, range 52 – 165), differences that were significantly different despite the small sample size ( $p = 0.018$ ; Kruskal–Wallace non-parametric test; Table 2). In contrast, no differences in frequency of diarrhoea or sampling location were observed between EV-G types (Table 2,  $p > 0.05$ ).

**Table 2.** Clinical characteristics of EV-infected pigs

Sample	Location	Age*	Diarrhoea	Ct value	EV-G Type
714133	Cao Lanh	3	No	25.5	1
724267	Chau Thanh	4	Yes	24.6	1
724268	Chau Thanh	4	Yes	28.1	1
724266	Chau Thanh	4	Yes	30.1	1
734041	Thanh Binh	7	Yes	27.3	6
734042	Thanh Binh	7	No	30	6
734148	Thanh Binh	7	No	30.7	6
714318	Cao Lanh	7	No	29.9	Unk/ND
714432	Cao Lanh	7	No	31.5	6
734006	Thanh Binh	9	Yes	29.6	1
744100	Hong Ngu	9	Yes	31.1	6
744101	Hong Ngu	9	No	28	6
734007	Thanh Binh	9	Yes	29.2	Unk/ND
734123	Thanh Binh	52	Yes	25.9	10
744032	Hong Ngu	56	No	29.9	6
744257	Hong Ngu	104	No	28.5	11
724118	Chau Thanh	165	No	29.6	8
724167	Chau Thanh	Unk	No	30.5	1
724162	Chau Thanh	Unk	Yes	30.3	9
724143	Chau Thanh	Unk	No	28.9	Unk/ND

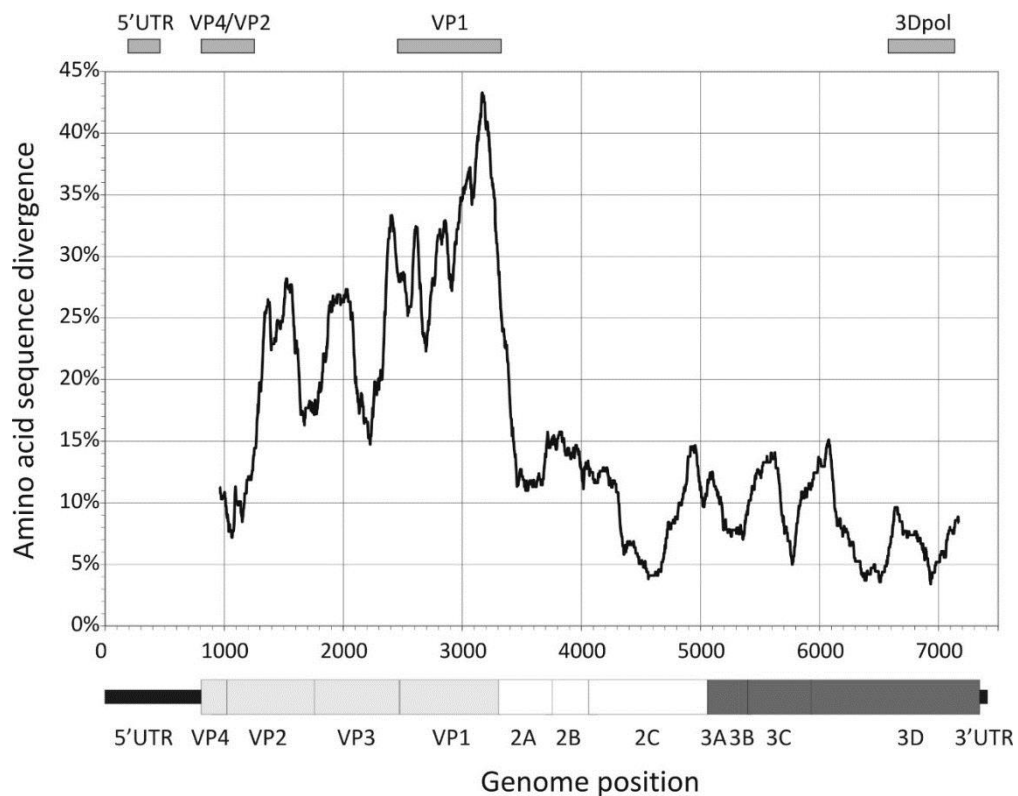
ND, Not determined; Unk, Unknown. \*Age in weeks; samples ranged by age.

### Recombination in the EV-G genome

The occurrence of recombination in picornavirus genomes is variable, even between closely related viruses in the same genus. We previously hypothesized that the generation of recombinants was favoured in genome regions showing restricted genetic variability and with consequent greater likelihood of creating biologically compatible genome combinations (Simmonds, 2006). Through a sliding window comparison of complete genome sequences of EV-G, amino acid sequences divergence was greatest in the structural gene (VP4–VP1)-encoding region and much more restricted in the non-structural gene block and in the 5' untranslated region (5'UTR, Figure 2).

Distinct patterns of divergence and codon usage have been observed between picornavirus groups that undergo frequent recombination between structural and non-

structural gene regions and those where recombination is infrequent or undetected (Simmonds, 2006). EV-G sequences showed characteristics of the former, with mean pairwise distances between structural gene sequences of EV-G substantially higher than non-structural gene regions (0.23 compared with 0.09, 2.5 : 1). The structural gene region additionally showed a higher frequency of non-synonymous to synonymous substitutions ( $dN/dS = 0.20$ ) and more random codon usage (effective number of codons (ENc) of 55; Fares *et al.*, 2002) than non-structural genes ( $dN/dS = 0.09$ ; ENc of 51). Indeed, the divergence scan (Figure 2) was highly comparable to that observed in EV species A – C, each of which is documented to undergo frequent recombination between non-structural regions and the 5'UTR, and in species B, additionally with VP4 (Oberste *et al.*, 2004; Simmonds, 2006).

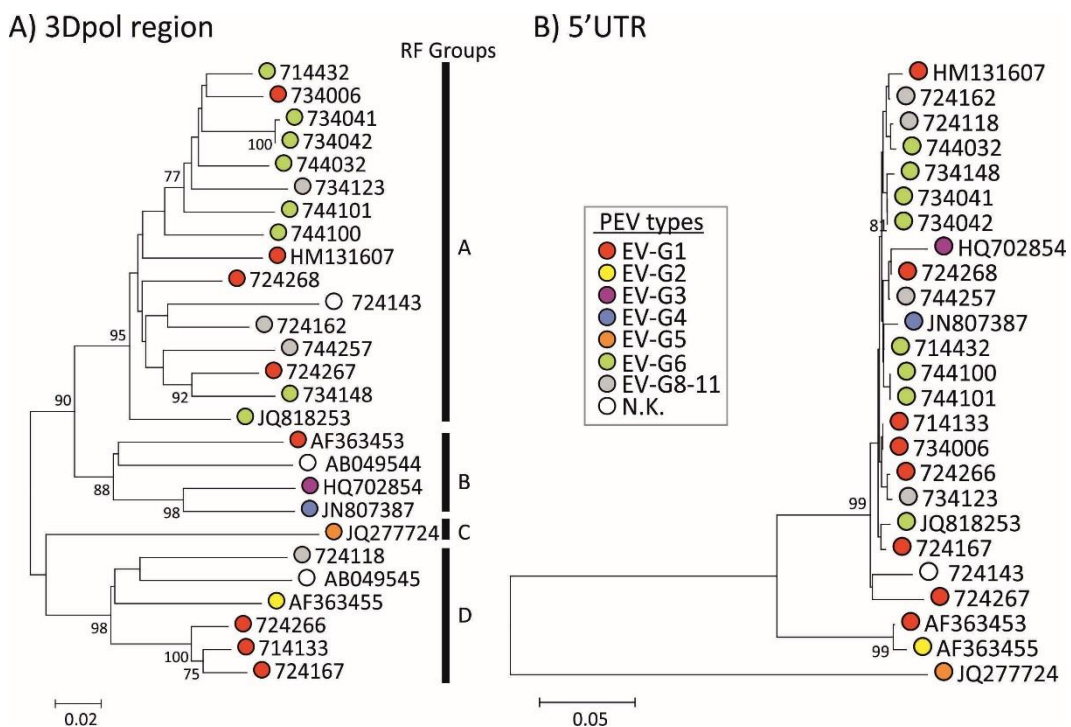


**Figure 2.** Amino acid sequence divergence across the genome of EV-G types 1 – 6. Divergence values were calculated using a window size of 300 bases, incrementing by a codon between windows. The genome diagram beneath is plotted to scale with the x-axis. Genome regions sequenced for recombination analysis (5'UTR, VP4/VP2, VP1 and 3Dpol) are shown above the graph.

Sequences from the 3Dpol region fell into four main bootstrap-supported clades on phylogenetic analysis of nucleotide sequences (Figure 3a). Membership of the four clades (designated A – D) bore little relationship to type assignments based on VP1



sequences; members of different types were found within each of the four clades, and conversely, sequences of the same type were also found in different clades. Pairwise distances within and between 3Dpol groups fell into two, largely distinct ranges (Figure S1, available in the online Supplementary Material) with a distance threshold matching clade membership of approximately 14%, comparable to that dividing recombinant forms in other EVs (McWilliam Leitch *et al.*, 2010, 2012). However, variants that were closely similar in VP1, such as the subcluster of three sequences 724167, 714133 and 734006, remained grouped together in 3Dpol (and in VP4/VP2), consistent with a degree of retained genetic linkage between the two regions over short periods of sequence divergence.



**Figure 3.** Phylogenetic comparisons of sequences from 3Dpol (nt 6585 – 7121) (a) and 5'UTR (nt 196 – 470) (b) from the study samples and available sequences of other EV-G variants from GenBank. Maximum-likelihood trees were constructed from nucleotide sequences with 500 bootstrap resamplings to demonstrate robustness of groupings; values of  $\geq 70\%$  are shown. The tree was drawn to scale; bar shows indicated evolutionary distance.

The phylogeny of VP1 and groupings of EV-G variants into types was mirrored in part in the VP4/VP2 regions but with some exceptions that were indicative of recombination between the two genome regions. The GenBank sequence HM131607 was assigned as EV-G1 based on its grouping with other type 1 variants in VP1 but it

adopted a different tree position in VP4/VP2. A more complex change in phylogeny occurs for the type 6 variants obtained in the current study; two of these (744032 and 744101) that cluster with type 6 in the VP1, cluster to form a new clade in VP4 that also includes the newly assigned EV-G10 variant, 734123.

The phylogeny of the 5'UTR was even less congruent with VP1 than VP4/VP2 (Figure 3b). All but three variants possessed 5'UTR sequences that were nearly identical to each other and fell into a separate bootstrap-supported clade that grouped separately from the prototype type 1 and type 2 sequences, GenBank accession nos AF363453 and AF363455. The most divergent 5'UTR sequence was JQ277724, a type 5 variant isolated from a sheep and proposed to be an interspecies recombinant virus with a 5'UTR most similar to those of EV-E and -F (Boros *et al.*, 2012b).

## DISCUSSION

This study provides evidence for a broad ecology of EV-G and highlights substantial genetic diversity and a propensity for intra-species recombination. High rates of EV-G infection were detected in young pigs; detection frequencies of over 90% in young pigs and 40% in adult pigs are consistent with 92 and 20% positivity rates in the equivalent age groups reported from the Czech Republic (Prodělalová, 2012) although substantially higher than in China (10 and 5%, respectively) (Yang *et al.*, 2013), Hungary (Boros *et al.*, 2011), Italy (Sozzi *et al.*, 2010) and Spain (Buitrago *et al.*, 2010). High rates of infection in young pigs likely reflect their exposure to environmental sources of infection and infection with potentially multiple types of circulating EV-G strains. Although numbers were relatively small, type 1 infections were specifically found in younger pigs (sucklers and weaners; 3 – 9 weeks) and type 6 infections were primarily detected in the age range 7 – 9 weeks, while all but one of the older pigs (age range 52 – 165 weeks) were infected with types 8 – 11. The observations are consistent with successive rounds of infection of pigs in Vietnam, starting with EV-G1, followed by EV-G6 and thereafter more sporadic infections with rarer types. This perhaps mirrors age-associated differences in infection frequencies of different EV serotypes in young children (Khetsuriani *et al.*, 2006). However, it is also possible that observed associations were the result of different sampling times in

different farms and districts; this age association requires confirmation with larger numbers.

We found no association between PEV detection and enteric disease (diarrhoea) in this cross-sectional study (Table 1). These findings are consistent with a previous study that clearly differentiated EV-G from more pathogenic porcine picornaviruses (Prodělalová, 2012). In this respect, EV-G infections resemble those of EV species infecting humans that are largely non-pathogenic, appear to be ubiquitous and frequently detected in the enteric and respiratory tracts of healthy people, but for which infections with particular variants may be associated with neurological syndromes. There is limited evidence for systemic infections of pigs with EV-G; one experimental infection study of specific-pathogen-free pigs showed detectable viral RNA in plasma, spinal cord and brain (Yang *et al.*, 2013), and three of 12 inoculated pigs developed flaccid paralysis of the hind limbs. These intriguing results suggest the possibility that certain EV-G types may be pathogenic. Unfortunately, the study did not provide the necessary sequence data to support this conclusion (the cited GenBank accession numbers were incorrect in the manuscript) and, indeed, evidence for pathogenic variants of EV-G has not been reported elsewhere in the literature, either from field epidemiology studies or from laboratory infection trials.

The genetic diversity of EV-G is incompletely characterized. Both prototype strains [EV-G1, originally described as PEV-9, and EV-G2 (PEV-10)] have been detected in recent surveys of domestic pig populations (Sozzi *et al.*, 2010; Ren *et al.*, 2012) and several further examples of EV-G1 were detected in the current study. A substantial proportion of our study group were infected with EV-G6, to date described as a single variant from Korea (Moon *et al.*, 2012). Reports of EV-G3, EV-G4 and EV-G5 are similarly sparse, in each case restricted to the single publications in which they were first described (Boros *et al.*, 2011, 2012a, b). The likelihood that viruses in species G are actually highly diverse genetically (and likely serologically) but incompletely sampled is supported by observations in the current study in which genetic analysis of a relatively small sample identified four new EV types (G8 – G11). Further evidence for the currently uncharted diversity of this EV species is provided by analysis of partial VP1 sequences published from previous surveys deposited in GenBank (La

Rosa *et al.*, 2006; Sozzi *et al.*, 2010; Nix *et al.*, 2013). Although sequences in these studies were too short for formal type assignments and were not suitable for uncovering robust phylogenetic relationships, two surveys of infected pigs in Italy (La Rosa *et al.*, 2006; Sozzi *et al.*, 2010) generated sequences potentially representing six new types, while a further potential new type was observed in Bolivia (Nix *et al.*, 2013). Larger scale and more systematic surveying of variants infecting pigs in different countries will undoubtedly reveal the existence of much larger numbers of EV-G types in the future.

Several differences in phylogeny relationships were observed between VP1 (used for type assignment) and other genome regions (3Dpol, VP4/VP2 and 5'UTR). Changes in phylogeny are, by analogy with the better documented human EVs, strongly indicative of recombination events occurring during EV-G diversification. EV-G shares with other EV species a number of genome properties, such as restricted amino acid sequence diversity in NS gene regions and a modularly functioning 5'UTR, that favour recombination by increasing the likelihood that a chimeric sequence will be biologically viable, as discussed previously in reviews by Simmonds (2006) and Lukashev (2005). Previous descriptions of recombination in EV-G are restricted to those involving the 5'UTR; Boros *et al.*, (2012b) identified a highly divergent 5'UTR sequence on an EV-G variant (EV-G5) that grouped with those from bovine EVs (EV species E, F). This probably represents an interspecies recombination event of the type documented previously for example between rhinovirus species A and C (Huang *et al.*, 2009; McIntyre *et al.*, 2010) and between EV species A and B (Santti *et al.*, 1999). Bovine EV 5'UTR sequences in species E and F are similarly interspersed phylogenetically. In a second study, the EV-G1 variant Ch-ah-f1 (GenBank accession no. HM131607) showed a change in phylogeny relationships in the 5'UTR with the prototype EV-G1 and G2 strains (GenBank accession nos AF363453 and AF363455) and the type 3 sequence (GenBank accession no. HQ702854). On analysis of the expanded dataset in the current study (Figure 3b), this finding is actually a manifestation of the separate clustering of the older AF363453 and AF363455 variants from the more recently detected EV-G variants, and not a recombination event specific to Ch-ah-f1. Remarkably, it appears that all existing types circulating in Europe and

Asia, including modern versions of EV-G1 and -G2, share a common 5'UTR sequence acquired in the period following the original isolation dates of the EV-G prototype strains (1973 for EV-G1 and 1975 for EV-G2 respectively; N. J. Knowles, personal communication).

Through the identification of additional phylogenetic incompatibilities in other genome regions, the current study additionally identifies extensive recombination between structural (VP1) and non-structural gene regions (3Dpol) and within the capsid (between VP1 and VP4/VP2). Membership of the four bootstrap-supported clades in 3Dpol indeed bears little relationship to their type assignments based on VP1 sequences (Figure 3a), while further, different, recombination events probably underlie the altered position of the type 1 variant, HM131607, and splitting of sequences from study samples assigned as EV-G6 into two clades in the VP4/VP2 region (Figure 1a). Clades in 3Dpol show a degree of divergence from each other comparable to those previously characterized in species A and B human EVs, and which were used to subdivide members of the same serotype into a series of recombinant forms (McWilliam Leitch *et al.*, 2010, 2012). In EV-G, however, these groups are not numerous, and individual 3Dpol clades contain examples of several different types; this is rarely observed in species A (EV71) or species B (echoviruses 9, 11 and 30) EVs. These differences reflect probable differences in transmission dynamics and reservoir sizes, and potentially differences in recombination frequency between species. Larger datasets are required to compare timescales of recombination events and recombinant form half-lives with those previously established for human EVs and parechoviruses (McWilliam Leitch *et al.*, 2009, 2010, 2012; Calvert *et al.*, 2010).

In conclusion, this study reveals the high frequency of infection with species G EVs in pigs in Vietnam, with almost universal infection among post-weaning pigs. EVG variants were highly genetically diverse and, although a relatively small number were analysed, suggest much greater genetic diversity within species G than has been characterized to date.

## **METHODS**

### **Study location and design**

The survey was carried out between February and May 2012 in Dong Thap province in southern Vietnam. The study included four of 12 districts (Cao Lanh, Chau Thanh, Hong Ngu and Thanh Binh) from which a total of 102 farms were randomly selected and sampled. From each farm, freshly voided individual faecal samples (~5 g) were randomly collected from 10 pigs, plus up to four samples per farm from any pigs with frank diarrhoea. Samples were recorded as diarrhoeic or not based on faecal consistency. Farmer survey questionnaires were used to collect information on animal and farm characteristics as well as farming practices. The study was approved and implemented by the Subdepartment of Animal Health Dong Thap province and veterinary students from Nong Lam University. A total of 198 faecal samples were subsampled from the total sample set, representing one to three randomly selected pigs per farm, including all of the 70 diarrhoeic samples.

### **Porcine EV screening**

RNA was extracted from 200 µl of 10% (w/v) faecal suspensions using a MagNA Pure 96 Viral NA small volume kit (Roche) and an automated extractor (Roche). The presence of PCR inhibitors and RNA quality control were assessed by spiking samples with an RNA internal extraction control (equine arterivirus) prior to extraction. The total RNA recovered (60 µl in nuclease-free water) was stored at -80°C until use. cDNA was screened for EVs by real-time reverse transcription (RT)-PCR using primers from the 5'UTR as described previously (Beld *et al.*, 2004). A total of 20 samples that were representative of the geographical range and ages of infected pigs were selected for further genetic analysis (type identification, recombination detection). These were re-extracted from the original specimens using Qiagen RNA extraction kits.

### **Amplification of VP4/VP2, VP1, 3Dpol and 5'UTR sequences**

These four genome regions were amplified by nested RT-PCR using primers listed in Table S1. For each PCR, 6 µl extracted RNA was used for cDNA synthesis followed by nested PCR. The reverse transcription step was combined with first-round PCR using Superscript III One-Step RT-PCR system with Platinum Taq High Fidelity according to the manufacturer's instructions (Invitrogen). The RT-PCR conditions were, sequentially, 43°C for 1 h and 20 cycles of 53°C for 1 min and 55°C for 1 min, followed by 70°C for 15 min and 94°C for 2 min. PCR comprised 40 cycles of 94°C (30 s), 50°C (30 s), and 68°C (105 s) and a final extension at 68°C for 5 min. After that, 1 µl of the first-round reaction was used for the nested PCR with second-round primers and GoTaq DNA polymerase (Promega). PCR amplification included 30 cycles of denaturation (at 94°C for 30 s), annealing (50°C, 30 s) and elongation (72°C, 90 s) in a thermal cycler. Products of the second-round PCR were visualized by agarose gel electrophoresis and sequenced.

Amplicons were sequenced directly using ABI 7200 BigDye capillary sequencing (Applied Biosystems) in a total volume of 10 µl containing 7 µl DNA/RNA-free water, 1 µl BigDye, 1 µl inner sense primer or antisense primer and 1 µl DNA amplicon. Reactions were carried out with 25 cycles of 30 s at 96°C, 20 s at 50°C and 4 min at 60°C.

Sequences were imported and aligned using the SSE sequence editor (Simmonds, 2012) and phylogenetic trees reconstructed using maximum-likelihood methods as implemented in the MEGA 5.2 software package (Tamura *et al.*, 2011). The optimum maximum likelihood model (lowest Bayesian information criterion score and typically greatest maximum-likelihood value) for each sequence dataset was first determined and used for phylogenetic reconstruction. Different models were selected for different datasets: Tamura–Nei gamma distribution (five rates selected) with invariant sites for VP4; general time-reversible gamma distribution (five rates) with invariant sites for VP1; Tamura–Nei, gamma distribution (five rates) for 3Dpol and Kimura two-parameter gamma distribution for the 5'UTR. However, running the datasets with second- or third-choice models created trees with identical topologies and with similar branch lengths and bootstrap values (data not shown). Phylogenetic analysis of each dataset used bootstrap resampling to determine the robustness of grouping.

Codon usage and pairwise distances were computed using built-in functions in the SSE sequence editor. All nucleotide positions were numbered using the annotation provided for the EV-G3 sequence, GenBank accession no. HQ702854 (swine/K23/2008/HUN).

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## **Large-scale screening and characterization of enteroviruses and kobuviruses infecting pigs in Vietnam**

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## **Large-scale screening and characterization of enteroviruses and kobuviruses infecting pigs in Vietnam**

### **ABSTRACT**

A recent survey of pigs in Dong Thap province, Vietnam identified a high frequency of enterovirus species G (*EV-G*) infection (144/198; 72.7%). Among these was a plethora of *EV-G* types (*EV-G1*, *EV-G6* and four new types *EV-G8* to *-G11*). To better characterize the genetic diversity of *EV-G* and investigate the possible existence of further circulating types, we performed a larger scale study on 484 pig and 45 farm-bred boar faecal samples collected in 2012, 2014, respectively. All samples from the previous and current studies were also screened for kobuviruses. The overall infection frequency of enteroviruses (*EV*) remained extremely high (395/484; 81.6%) but with comparable detection rates and viral loads between healthy and diarrhoeic pigs; this contrasted with less frequent detection of *EV-G* in boars (4/45; 8.9%). *EV* was most frequently detected in pigs  $\leq 14$  weeks old (approximately 95%) and declined in older pigs. Infections with *EV-G1* and *EV-G6* were most frequent, while less commonly detected types included *EV-G3*, *EV-G4*, *EV-G8* – *EV-G11* and 5 new types (*EV-G12* to *-G16*). In contrast, kobuvirus infection frequency was significantly higher in diarrhoeic pigs (40.9% compared to 27.6%;  $p = 0.01$ ). Kobuviruses also showed contrasting epizootologies and age associations; a higher prevalence was found in boars (42%) compared to domestic pigs (29%) with the highest infection frequency among pigs  $> 52$  weeks old. Although genetically diverse, all kobuviruses identified belonged to species *Aichivirus C*. In summary, this study confirms infection with *EV-G* was endemic in Vietnamese domestic pigs and exhibits high genetic diversity and extensive inter-type recombination.

### **INTRODUCTION**

Porcine enteroviruses (*PEV*) are members of the family *Picornaviridae* and originally consisted of 13 types (*PEV-1* to *-13*). Further studies on their genetic relatedness led to the re-assignment of these viruses into three separate picornavirus genera, *Enterovirus*, *Sapelovirus* and *Teschovirus* (Kaku *et al.*, 2001; Knowles, 2006;

Krumbholz *et al.*, 2002; Zell *et al.*, 2001). Of these, PEV-9 and -10 were classified as EV-G1 and -G2 serotypes in *Enterovirus G* with the subsequently characterised EV-G types EV-G3 through EV-G6 reported in single publications from Hungary and South Korea (Boros *et al.*, 2012a; Boros *et al.*, 2012b; Boros *et al.*, 2011a; Moon *et al.*, 2012). EV-G7 was subsequently discovered from domestic sheep by Boros and Knowles (unpublished). More recently, our molecular characterization of enteroviruses in a small number of PCR-positive faecal samples from domestic pigs identified a further four types (EV-G8 to -11) within this species. The presence of such a large amount of genetic diversity within such a modest number of samples sizes suggests that the full range of EV-G types in this virus species has yet to be fully characterised (Nguyen *et al.*, 2014a). In contrast to teschoviruses, infections with species G enteroviruses have not been linked to any enteric or other disease presentations (Knowles, 2006) except for skin lesions (Knowles, 1988) and flaccid paralysis in an experimental infection of pigs in China (Yang *et al.*, 2013). Viruses in this species have been reported from both healthy and diarrhoeic pigs with no statistically significant difference in infection rates between the two groups (Nguyen *et al.*, 2014a).

Porcine kobuvirus (PKV) was first identified in 2008 in Hungary (Reuter *et al.*, 2008). While screening 15 faecal samples from pigs for calicivirus (norovirus and sapovirus) using reverse transcription-PCR, Reuter *et al.* (2008) detected, in addition to a band specific for sapovirus, a nonspecific, strong band of about 1.1 kb in all tested samples visible on agarose gel. The nucleotide sequence of the nonspecific PCR product showed most similarity to the 3C/3D region of bovine and human aichiviruses. Upon analysis of the obtained complete nucleotide sequence, the new PKV was assigned as a member of a new species (*Aichivirus C*) in the genus *Kobuvirus*, which also includes *Aichivirus A* and *Aichivirus B* species, originally detected in humans and oxen, respectively. *Aichivirus C* consists of a single type, PKV-1. The existence of PKV-1 in pigs has been well documented in different countries worldwide (Amimo *et al.*, 2014; Barry *et al.*, 2011; Chen *et al.*, 2013; Di Bartolo *et al.*, 2015; Fan *et al.*, 2013; Khamrin *et al.*, 2010; Khamrin *et al.*, 2009; Okitsu *et al.*, 2012; Park *et al.*, 2010;

Reuter *et al.*, 2013; Sisay *et al.*, 2013) but in Vietnam, PKV-1 has been only reported from river water (Inaba *et al.*, 2014).

We have performed this larger scale screening study to better understand of the prevalence, genetic diversity and disease association of enteroviruses and kobuviruses infecting pigs and farm-bred boars (hereafter referred to as ‘boars’) in Vietnam.

## RESULTS

### Detection of EV

EV RNA was detected in all but one (103 of 104; 99%) of farms containing domestic pigs and in 3 from 6 (50%) farms housing boars. 395 from 484 (81.6%) samples from domestic pigs and 4/55 (8.9%) of boar samples were PCR-positive (Table 1). Pigs of 14 weeks of age or younger were the most frequently infected with faecal detection frequencies of nearly 95%. Lower detection frequencies were observed in older pigs with the lowest rate (50.5%) in pigs > 1 year of age. Combining screening data with that of our previous samples, we found no significant difference in the frequency of detection of EV infection between the two groups in all ranges of age (Table 1). The lack of any direct causative role of EV-G in enteric disease in pigs was further demonstrated by our observation that pigs with diarrhoea showed slightly lower EV-G RNA viral loads than found in healthy pigs ( $p = 0.008$ , Mann-Whitney test; Figure 1).

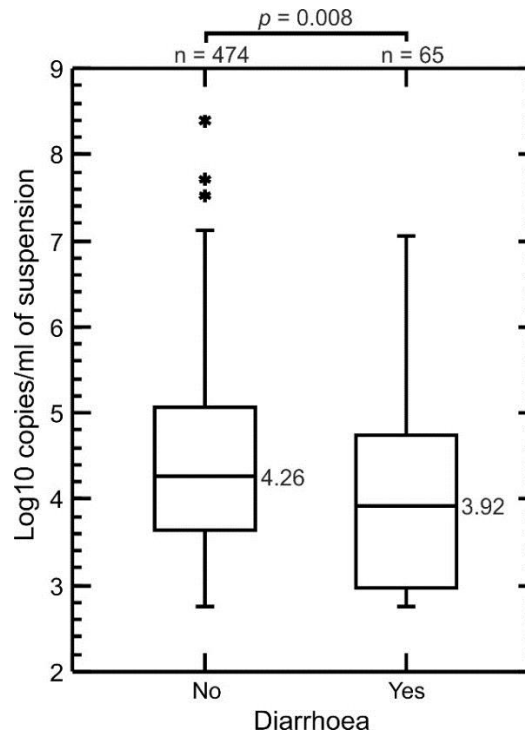
**Table 1.** Detection of EV and PKV in pigs

	Age (weeks)	Pigs tested*	EV		PKV		EV, PKV co-infection (%)*	<i>p</i> -value <sup>†</sup>
			Prevalence (%)*	<i>p</i> -value <sup>†</sup>	Prevalence (%)*	<i>p</i> -value <sup>†</sup>		
Suckler	< 7	11/78	90.9/93.6	0.56	63.6/59	1	63.6/53.8	0.75
Weaner	7 – 14	32/147	93.8/94.6	1	18.8/25.9	0.4	18.8/24.5	0.49
Grower	15 – 23	8/117	75/82.9	0.63	25/16.2	0.62	25/13.7	0.32
Gilts	24 – 52	0/68	-/70.6	-	-/26.5	-	-/17.6	-
Matured pigs	> 52	28/106	39.3/48.1	0.4	<b>67.9/25.5</b>	<b>2.7x10<sup>-5</sup></b>	28.6/13.2	0.08
	Total	79/516 <sup>‡</sup>	72.2/79.1	0.17	<b>43/28.7</b>	<b>0.01</b>	29.1/23.3	0.26

\* Data were presented in pair of diarrhoeic/non-diarrhoeic samples.

<sup>†</sup> Fisher exact test or Chi-squared test

<sup>‡</sup> Only samples from the previous and current studies with available data (age, clinical status) were included in this table.



**Figure 1.** Box plot of log<sub>10</sub> EV RNA copies/ml of EV-G screen positive sample suspensions from healthy and diarrhoeic pigs. Median values are shown. Mann-Whitney test was applied to compare medians between groups.

### Detection of kobuviruses

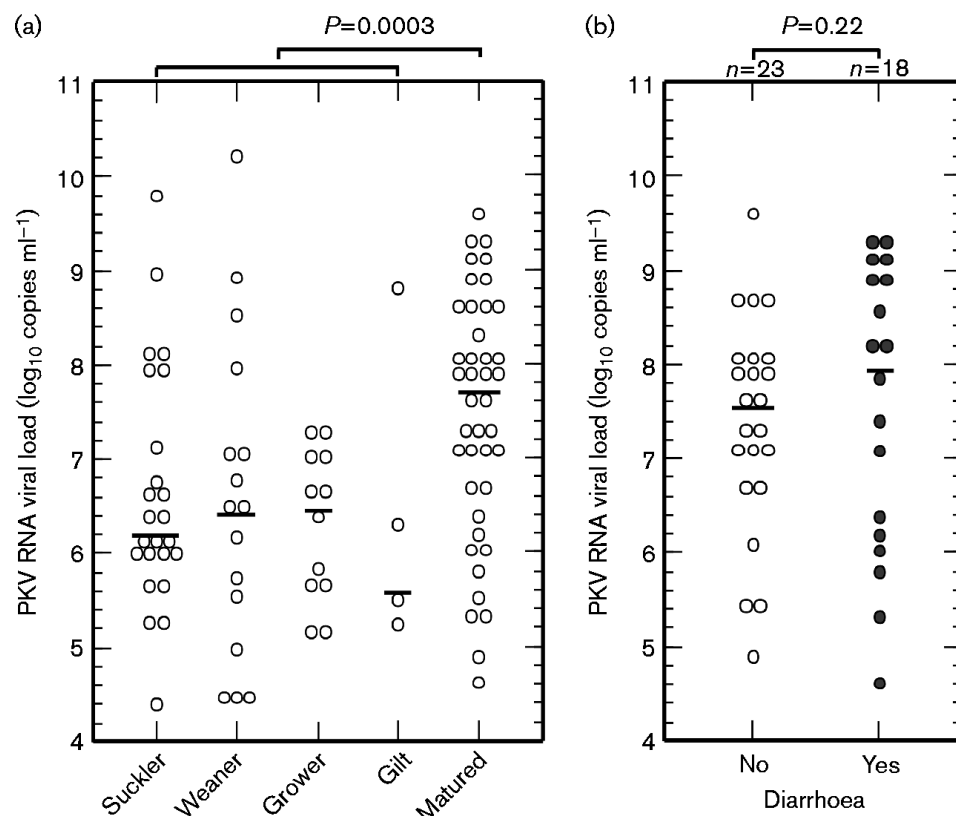
The overall detection frequencies of kobuviruses in domestic pigs and boars were 29.3% (200/682) and 42.2% (19/45), respectively. In contrast to *EV-G*, kobuviruses were significantly associated with diarrhoea in pigs, with an infection frequency of 40.9% (36/88) in diarrhoeic pigs and an infection frequency of 27.6% (164/594) in asymptomatic pigs ( $p = 0.01$ ; Chi-squared test). This positive association was, however, most marked in pigs aged  $> 52$  weeks at which the highest prevalence (67.9%) was recorded. For pigs  $\leq 23$  weeks old, infection frequencies were highest in sucklers and generally decreased with age.

In order to elucidate whether PKV viral loads were associated with clinical status of pigs, a real-time PCR protocol was developed. The assay had a detection limit of 100 PKV RNA copies per reaction and an amplification efficiency of 89% (data not shown). It showed good specificity (no false positive in 20 samples negative by nested PCR) and sensitivity (97/100 samples positive by nested PCR were detected by real-time PCR). The median log<sub>10</sub> viral load (7.7) of PKV in 41 matured pig samples was



significantly higher than that (6.4) in the group of 56 younger pig samples ( $p = 0.0003$ ; Mann-Whitney test; Figure 2a). However, there was no significant difference in the viral loads (medians of  $\log_{10}$  viral loads of 7.99 and 7.56) between matured pigs with and without diarrhoea ( $p = 0.22$  by Mann-Whitney test; Figure 2b).

The differences in the coinfection rates were not significant in all categories of age between the two groups of diarrhoeic and non-diarrhoeic pigs ( $p > 0.05$ ). Co-infection with EV and PKV was most common in youngest pigs (Table 1).

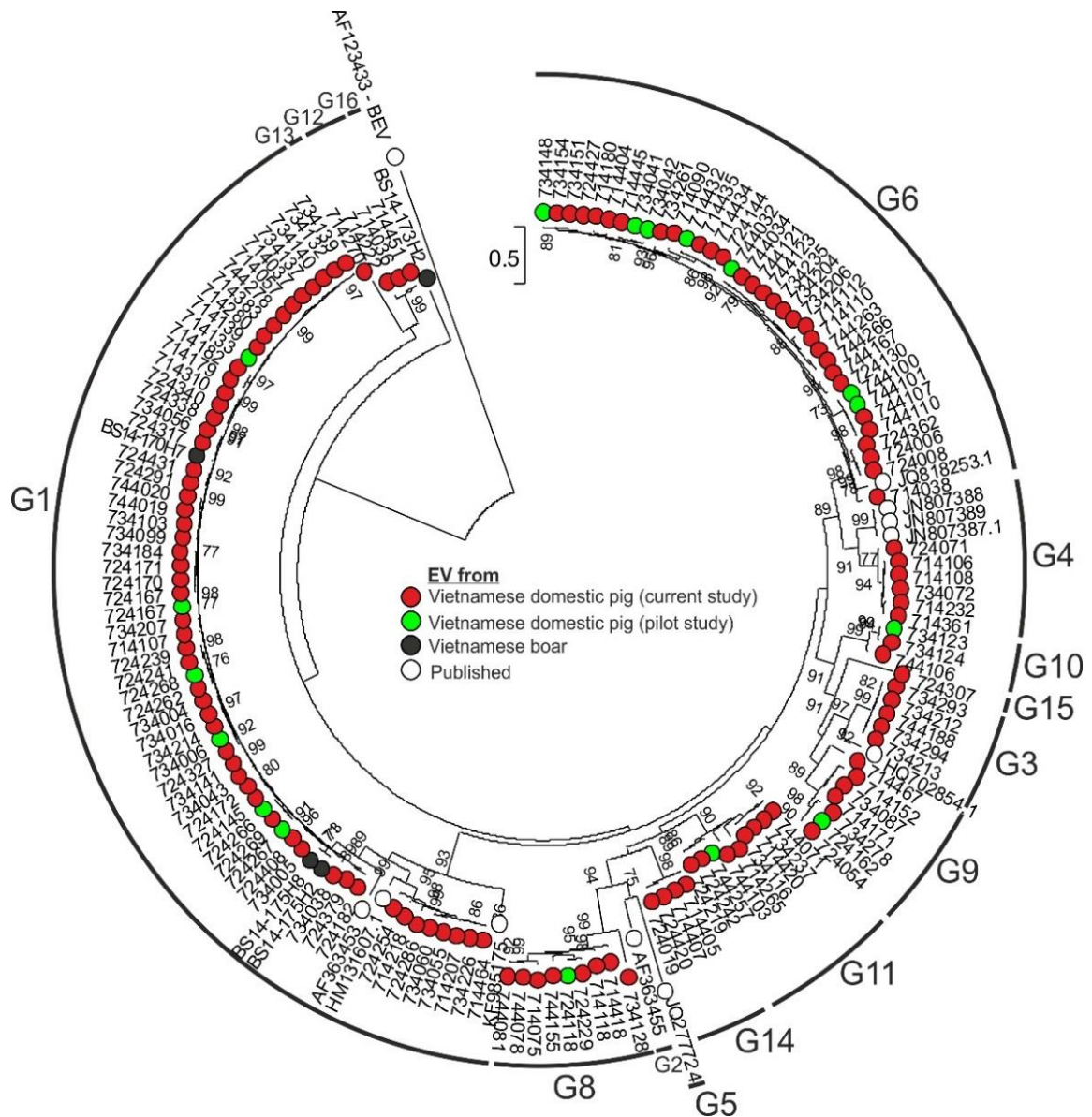


**Figure 2.**  $\log_{10}$  PKV RNA copies/ml of pig sample suspensions in different age groups (a) and in healthy or diarrhoeic matured pig (b). Bars show median values. Mann-Whitney test was applied to compare medians between groups.

### Genetic characterization of porcine EV

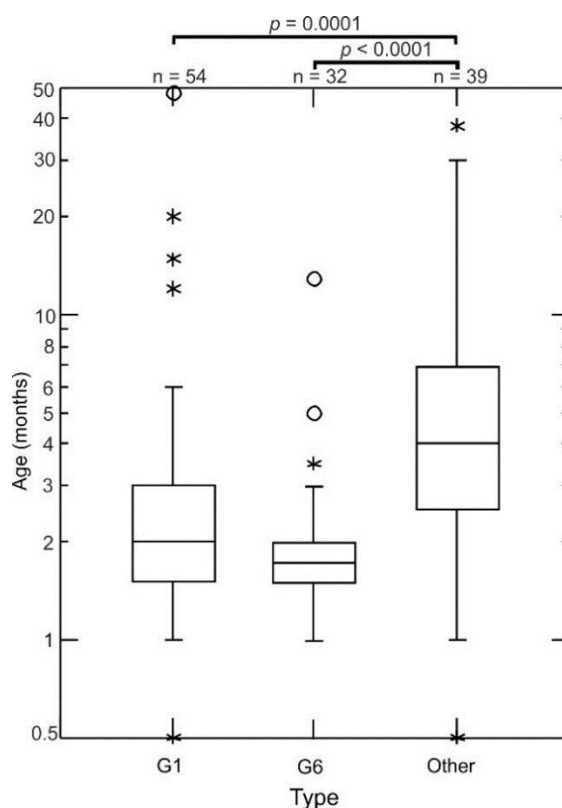
A range of EV variants in a subsample of 177 positive samples representing all sampling districts, Ct value ranges, ages and clinical status of infected pigs, and four boar positive samples were typed by sequencing of the VP1 region. All VP1 sequences obtained from 130 samples (including four from boars) clustered with the species G enteroviruses (Figure 3). Phylogenetic and sequence distance analysis of this region

showed the most commonly identified previously described type was EV-G1 ( $n = 57$  sequences, 3 boar-derived), followed by EV-G6 ( $n = 29$ ), EV-G11 ( $n = 9$ ), EV-G4, EV-G8, EV-G9 (6 of each type), EV-G3 ( $n = 5$ ) and EV-G10 ( $n = 2$ ). The other 10 VP1 nucleotide (nt) sequences were substantially divergent ( $> 25\%$ ) from other EV-G types and have been assigned as new types EV-G12 to EV-G16 by the ICTV Picornavirus Study Group (Figure 3).



**Figure 3.** Phylogenetic comparisons of VP1 sequences (nt 2469 – 3317) from study samples and available sequences of other EV-G variants from GenBank. Maximum-likelihood trees were reconstructed using 1000 bootstrap resamples to demonstrate the robustness of groupings; bootstrap support values of  $\geq 70\%$  are shown. The tree was rooted using bovine enterovirus (BEV), GenBank accession no. AF123433, as an outgroup. The tree was drawn to scale; bar indicates estimated number of substitutions per site.

Analysis of data shows that young pigs were more frequently infected with EV-G1 and EV-G6 compared to other EV-G types, with median ages of 2 and 1.75 months, respectively. Meanwhile, other types were generally found in older pigs (median age: 4 months) (Supplementary data; Table S3). These differences were statistically significant ( $p < 0.0001$ ,  $p = 0.0001$  by Mann-Whitney test; Figure 4).

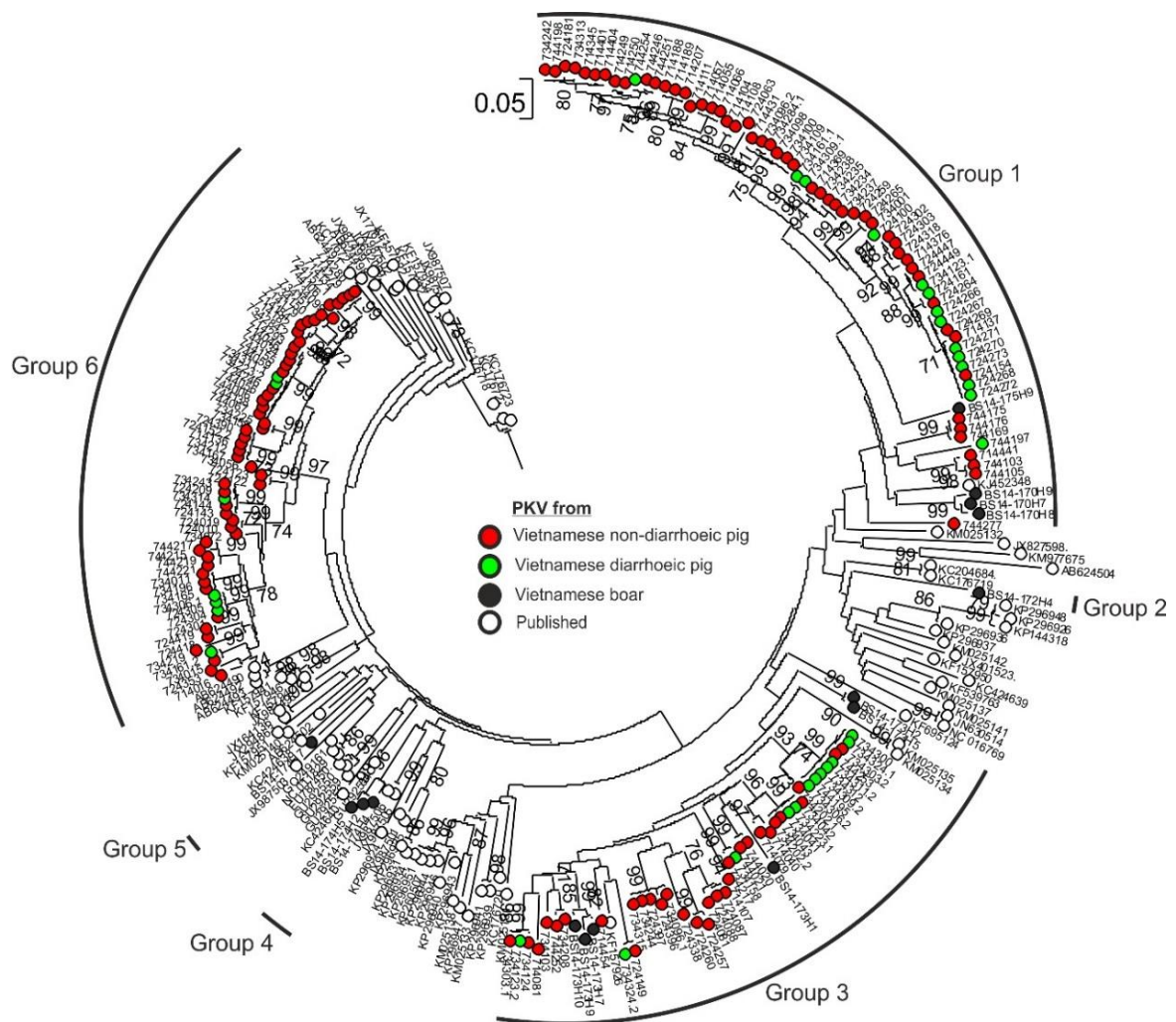


**Figure 4.** Box plot of EV types by pig ages. The y-axis is displayed in a log10 format. Bars show median values. Mann-Whitney test was applied to compare medians between groups.

### Sequence and phylogenetic analysis of PKV

184 VP1 sequences were obtained from 173 positive samples, including 15 from boars (Figure 5). Co-infection with two kobuvirus variants was detected in 11 samples by sequencing of VP1. All the VP1 sequences in the current study clustered within species *Aichivirus C* with nucleotide and amino acid sequence distances ranging from 0 – 22.3% (mean = 14.3%) and 0 – 17.6% (mean = 8.5%), respectively. In comparison to VP1 sequences of *Aichivirus C* available in GenBank, the novel sequences produced here showed 9.1 – 23.6% and 0.45 – 17.5% nucleotide and amino acid distances, respectively. In the resulting phylogeny, sequences from Vietnam were segregated into

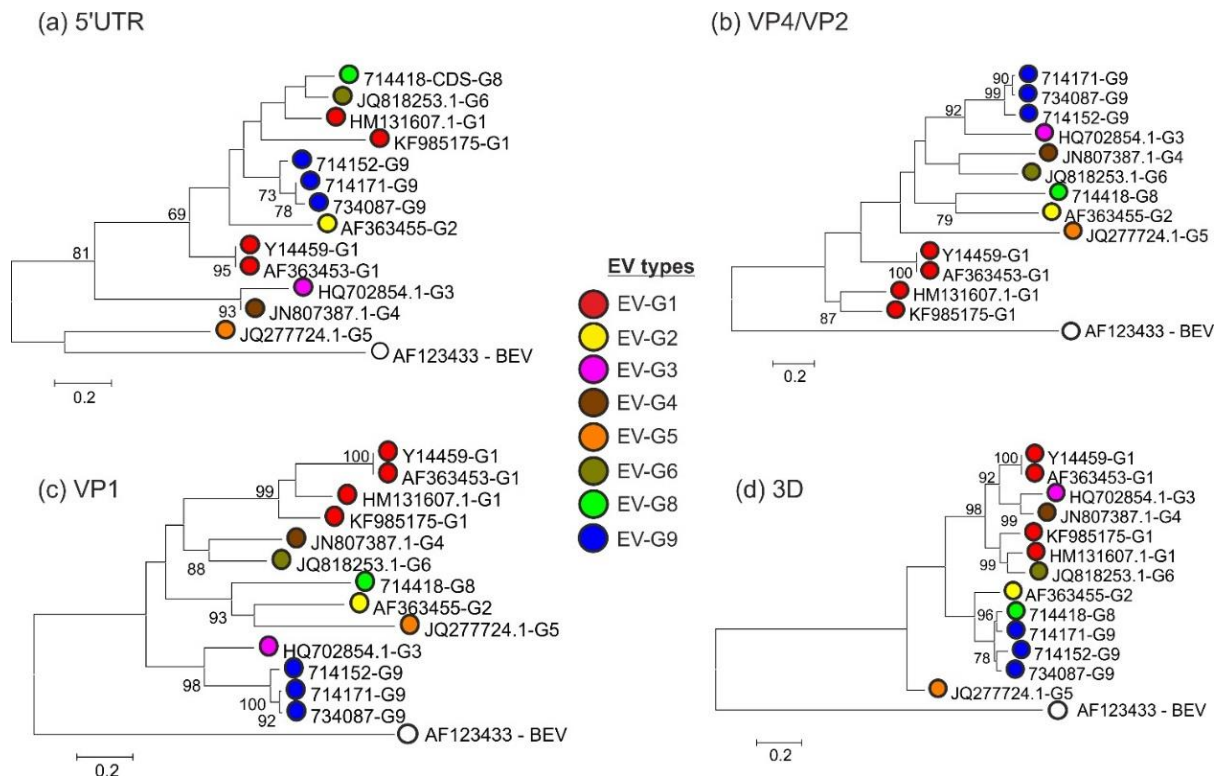
six groups (1 – 6) with sequences from boars representing five distinct lineages, while those from domestic pigs clustering in three large groups. Groups 1, 2, and 3 appeared to be most closely related to sequences from China (KJ452348, KF539763, KF157926), while groups 4, 5 and 6 were most closely related to sequences from USA (JX987506), Hungary (GQ249161.1) and Thailand (AB624490), respectively. To test whether specific groups were associated with diarrhoea, association index values were calculated using genetic groups 1, 3 and 6 for group assignments and diarrhoea/non-diarrhoea as the associated variable. The calculated association index values of 0.45, 0.85 and 0.66 for the three groups, respectively, provided no evidence for difference in clinical outcomes between groups.



**Figure 5.** Phylogenetic comparisons of VP1 sequences (nt 3005 – 3838) from study samples and available sequences of other PKV variants from GenBank. The tree was rooted using human kobuvirus (NC 001918) and bovine kobuvirus (GU245693) as outgroups. The tree was drawn to scale; bar indicates estimated number of substitutions per site.

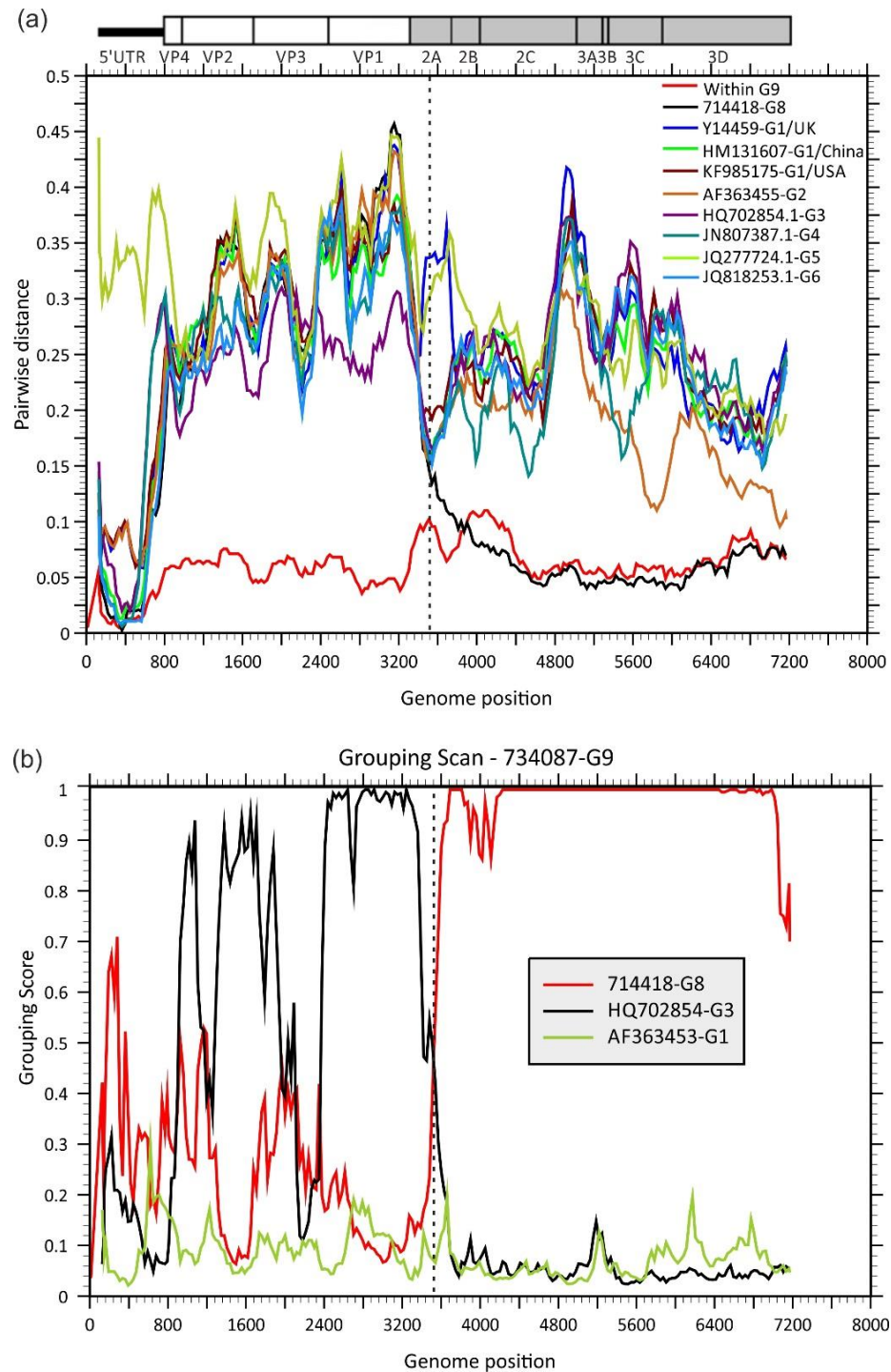
### Recombination of new types EV-G8, 9

To perform a recombination analysis, coding region complete sequences of two new types were obtained by overlapping PCR amplifications. Phylogenetic relationships between EV-G8 and EV-G9 and other EV-G types for which complete genome sequences were available were inconsistent in several different regions of the genome (5'UTR, VP4/VP2, VP1 and 3D), indicative of recombination (Figure 6). Most obviously, while sequences from the 5'UTR and VP4/VP2 and VP1 regions of the two novel EV-G types (EV-G8 and G9) clustered separately from all other known EV-G types, sequences from the 3D regions of these novel viruses clustered together and formed a well-supported monophyletic clade (Figure 6). To identify sites where recombination is likely to have occurred, a nucleotide distance scan (Figure 7a) and grouping scan (Figure 7b) were performed using EV-G9 as the reference sequence. G9 was equally divergent from G8 in P1 as it was from other types, but divergence values between G8 and G9 plunged at the boundary of P1/P2 to values equal to or lower than the mean pairwise distance of sequences within EV-G9. The analysis localised the breakpoint to the middle of the 2A region.



**Figure 6.** Phylogenetic comparisons of sequences from 5'UTR (nt 107 – 809) (a), VP4/VP2 (nt 810 – 1752) (b), VP1 (nt 2469 – 3317) (c), and 3D (nt 5934 – 7316) (d) from the study samples and available sequences of other EV-G variants from GenBank.





**Figure 7.** (a) Nucleotide sequence divergence within EV-G9 and between EV-G9 sequences and other EV-G types. Y-axis values show mean pairwise distances between 300 base windows across the genome, incrementing by 30 bases between fragments. (b) Group Scan analysis of the sequence 734087-G9. Sequence fragments of 300 bases incrementing by 30 bases, 100 bootstrap replicates, were compared with sequences from other types. To show gene boundaries, the P2 & P3 regions have been shaded grey and a scale representation of gene positions in the EV polyprotein shown above graphs.

## DISCUSSION

This is the first large scale study of the prevalence and genetic diversity of enteroviruses and kobuviruses in pigs and boars, providing substantial new information on their high infection frequencies and co-circulation of multiple types and lineages of both viruses in domestic pig and boars in Vietnam.

The overall detection frequency of EVs in domestic pigs (81.6%) was notably higher than those reported in Spain (0%), Italy (7.5%), China (8.3%) and the Czech Republic (50.2%) (Buitrago *et al.*, 2010; Prodělalová, 2012; Sozzi *et al.*, 2010; Yang *et al.*, 2013). In contrast, detection of EV RNA in boars in this study was much lower than the 50% and 69.4% prevalence reported in Hungary (Boros *et al.*, 2012a) and the Czech Republic (Prodělalová, 2012), respectively. High rates of EV infection in young pigs (detection rate > 90%) suggested that these viruses maintain endemic transmission in this population. Infection rates declined with the age of the pigs, to 50.5% in matured pigs, consistent with a limited period of active infection by and faecal excretion of circulating EV-G types, followed by clearance and likely immunity to reinfection. The greater likelihood of encountering the most actively circulating EV-G types (1 and 6) early in life may account for their increased detection in younger pigs (Figure 4). Because of the more limited circulation of other EV-G types, first exposure may be delayed and therefore account for the increased age range of pigs in which these types are detected.

Even though domestic pigs and boars are typically reared separately, we found that EV-G1 infected both animal populations. Furthermore, variants were phylogenetically interspersed, indicating transmission events between the two populations. How this occurs is currently unclear as the boars were sampled in farms without domestic pigs and *vice versa*.

Our study provided no evidence that infections with EV-G caused diarrhoea, with comparable detection frequencies in pigs with and without symptoms (Table 1), consistent with a previous study (Prodělalová, 2012). To date, only G1 virus has been specifically associated with disease in pigs, based on observations from the experimental infection of pigs in China in which two of twelve two week-old-specific-

pathogen-free pigs exhibited flaccid paralysis of the hind limbs with EV RNA detected in plasma, spinal cord and brain. In that experiment, the sample supernatant (not EV isolate) was filtered through 0.22 µm microfilters before inoculation (Yang *et al.*, 2013; Zhang *et al.*, 2012). The sample was negative for a number of pathogens in pigs (e.g. porcine teschoviruses, porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, classical swine fever virus, porcine epidemic diarrhoea virus, porcine rotavirus, hepatitis E virus). However, findings from our study indicate that this must be a rare outcome of infection given the absence of any evident neurological disease in pig farms where EV-G1 (and other enterovirus types) circulate extensively.

### **Genetic diversity and recombination in EV-G**

Findings of four new types (G8 – G11) from only 20 positive samples in our previous study suggested that the genetic diversity of species G enterovirus had not been completely characterized (Nguyen *et al.*, 2014a). This conclusion was supported by the detection of five more novel types from 130 positive samples in the current study. There is no doubt that more new EV-G types will continue to be detected from pigs from different countries in the future. To further characterize the genetic diversity of EV-G and potential occurrence of recombination between types, almost complete genome sequences of EV-G8 and EV-G9 were obtained and compared to those of other EV-G types. Collectively, EV-Gs show generic characteristics, such as patterns of divergence, codon usage, non-synonymous to synonymous substitutions that occur in other picornaviruses prone to extensive recombination, including EV species A – C (Nguyen *et al.*, 2014a; Oberste *et al.*, 2004b; Simmonds, 2006). Consistent with these predictions, recombination in the EV-G genome was detected, with breakpoints located at the border of the 5'UTR and VP4 of the EV-G5 prototype sequence (Boros *et al.*, 2012b). Our previous study additionally identified other recombination occurring in the HM131607 – G1 genome and other enterovirus sequences from domestic pigs in Vietnam (Nguyen *et al.*, 2014a). In the current study, sequences of EV-G8 and G9 variants showed significant phylogenetic incongruence between 3D (in which G8 and G9 sequences grouped together) and other regions (5'UTR, VP4/VP2 and VP1 – where G8 clustered separately from G9 sequences). This inconsistency in



tree topology is strongly suggestive of recombination. Fragments 3 (nt 2395 – 3390) and 4 (nt 2679 – 5419) of the G9 variants, both amplified by a single PCR (Figure S1), had a long identical overlapping region that was distinct from the corresponding region of G8. This helped rule out the possibility of hybrid viruses formed by assembly error from co-infection of individual pigs with two or more EV variants. When comparing the G8 and G9 variants, a nucleotide sequence divergence scan (Figure 7a) showed considerable divergence in P1; from the middle of 2B to the end of the coding region, however, divergence declined substantially to levels even lower than that observed within G9 sequences alone. The grouping scan (Figure 7b), which quantifies degrees of grouping with pre-defined groups (types), similarly identified a recombination breakpoint in the middle of the 2A region.

### **Infection frequencies, diversity and disease associations of PKV**

In accordance with previous studies (Chen *et al.*, 2013; Park *et al.*, 2010), our study showed an association between PKV detection and the presence of diarrhoea in matured pigs (Table 1), indicating its potential aetiological role in gastrointestinal (GI) disease, at least in older pigs. In marked contrast to EV infections, however, the highest detection rates were observed in diarrhoeic pigs > 52 weeks old (matured pigs). This contrasts with reports from other countries where pigs younger than 6 months, particularly sucklers, were most susceptible to PKV infection (Chen *et al.*, 2013; Park *et al.*, 2010). Furthermore, we found viral loads of PKV in matured pigs to be significantly higher than that in the younger pigs.

This observation of increasing detection frequencies with age may simply reflect the possibility that PKV circulates less extensively than other enteric viruses, such as EV-G, and that infections are skewed towards older age groups with greater cumulative exposure. Another hypothesis to explain increasing detection frequencies and greater viral loads in older animals is that PKV infections are naturally persistent, and pigs may sequentially acquire long-term infections with one or more PKV variants over time. Although frequencies of persistence in PKV and other kobuviruses are poorly documented, genomes of all members of this genus possess structured RNA genomes (Davis *et al.*, 2008a; Simmonds *et al.*, 2004), a generic property associated with

persistence in several other picornavirus genera and among other families of positive-stranded RNA viruses. High rates of detection in older pigs and boars without diarrhoea and the lack of association between viral load and symptomatic infection in matured pigs indeed questions a direct aetiological link between PKV and GI disease. It is possible, for example, that the greater detection frequency of PKV in cases of diarrhoea was secondary to altered mucosal immunity and inflammatory processes consequent to bacterial or other viral infections causing diarrhoea (Frémont *et al.*, 2013). These processes may serve to re-activate normally relatively quiescent infections with PKV. Consistent with this possible incidental association with GI disease, comparison of the phylogeny of VP1 sequences from diarrhoeic and non-diarrhoeic pigs provided no evidence for the existence of specific lineages associated with GI disease. Formal testing of the association of this trait with phylogenetic position by calculation of association index values (Wang *et al.*, 2001) confirmed this lack of association.

VP1 sequences of PKV variants clustered in six genetically diverse groups that showed the closest relatedness to sequences from China, USA, Hungary and Thailand. VP1 contains a number of neutralization domains and the gene encoding this protein has been extensively used for (sero)type identification. *Aichivirus C* is currently classified as a single type, PKV-1, which includes variants with uncorrected VP1 nucleotide sequence distances ranging from 0 – 22.6%. There is no information on the extent of antigenic, neutralization or protective immunity differences between variants of PKV, or whether members of different genetic groups identified in VP1 correspond to serotypes of other enteroviruses. By analogy with other EVs, enterovirus species A – D (sero)types show > 25% nucleotide sequence distances from each other in VP1 (Oberste *et al.*, 1999) while different rhinovirus serotypes in the same genus indeed show divergences of  $\geq 12 - 13\%$  (McIntyre *et al.*, 2013b). As PKV VP1 sequence distances lie between these two thresholds, prediction of their serological properties using this type of comparative approach is problematic. However, the detection of different PKV variants in the same samples is more consistent with them being antigenically distinct and lacking cross-protection, comparable to what is observed

with different serotypes of PV and foot and mouth disease virus (Alexandersen & Donaldson, 2002; Alexandersen *et al.*, 2002).

**Conclusions.** This large-scale study showed high prevalence and genetic diversity of EV-G and PKV in pigs and boars in Vietnam. These are clearly widely distributed, with endemic infections circulating in both domestic pigs and boars, and demonstrate the ease with which enteric viruses likely transmitted by faecal/oral routes can become established in and transmit within farmed animal populations. While the pathogenicity of neither virus is clearly established in either this or previous studies, further genetic and epizootiological characterization of these viruses will contribute to our understanding of their transmission patterns and the farming practices that affect their spread. Future studies may also provide new insights into the factors that underlie the transmission and dissemination of more specifically pathogenic viruses, such as rotaviruses, that present a much greater challenge to animal welfare and productivity.

## **MATERIALS AND METHODS**

Faecal samples were collected from 104 farms in four districts of Dong Thap province between February and May 2012. At each farm, 10 freshly voided samples from randomly selected healthy pigs and up to four samples from pigs with diarrhoea were collected. From this collection, a subsample of 198 samples, including 70 diarrhoeic samples, were screened in a previous study (Nguyen *et al.*, 2014a). In the current study, we extended the screening of enteroviruses to the 18 remaining diarrhoeic samples and 466 samples representing three to five healthy pigs per farm. In addition, 45 faecal samples collected in April 2014 from healthy farm-bred boars that were housed in typical livestock pens on six farms in Dak Lak province were also included. All 727 samples from pigs and boars were also screened for kobuviruses.

**Screening and typing of enteroviruses.** RNA was extracted from faecal samples using a MagNA Pure 96 Viral NA small volume kit (Roche) and an automated extractor (Roche) as described previously (Nguyen *et al.*, 2014a). Reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen, UK) was performed according to the manufacturer's instruction. Enteroviruses were screened by a real-time PCR protocol (Beld *et al.*, 2004). Typing of enteroviruses from 177

positive samples, including 10 from diarrhoeic pigs, representing ranges of geography, Ct values and ages of infected pigs, was performed by amplification and sequencing of VP1 region as described previously (Nguyen *et al.*, 2014a). VP1 products of the nested PCR were sequenced on both strands using the BigDye Terminator v3.1 (Applied Biosystems) and primers for second round PCR.

**Screening and characterization of kobuviruses.** Synthesized cDNA was screened for kobuviruses by nested PCR using primers targeting the 3D region of all known kobuviruses (Supplementary Data; Table S1). The conditions for amplification (using GoTaq DNA polymerase from Promega, UK) were 95°C for 5 min, 30 cycles of denaturation (94°C, 30 s), annealing (54°C, 30 s), and elongation (72°C, 45 s). Kobuviruses in positive samples were characterized by amplification and sequencing of VP1 region with similar amplification conditions for enterovirus VP1 as described previously (Nguyen *et al.*, 2014a) but using an annealing temperature of 58°C in both rounds.

**PKV viral load measurement.** Using PKV sequences available from GenBank, primers and a probe targeting the 5'UTR region were designed to develop a real-time PCR protocol for measurement of PKV viral load. The 200 bp target region in the 5'UTR amplified from a positive sample was cloned into pGEM-T Easy Vector (Promega, UK). The linearised recombinant plasmid was used as template for transcription using MEGAscript T7 transcription kit (Ambion, UK) as previously described (McLeish *et al.*, 2012). RNA transcripts were used to assess the detection limit and efficiency of the assay before application on a subsample of 100 positive samples. These samples were selected to represent healthy and diarrhoeic pigs of all age groups, with focus on the group with disease association (matured pigs). The specificity of the assay was checked with 20 randomly selected negative pig samples that were negative for PKV but positive for EV. Each reaction of 20 µl total volume contained primers (PKV\_332s, PKV\_533a), probe (PKV\_505a\_probe) at final concentrations of 0.4 µM and 0.2 µM, respectively, cDNA (5 µl) and 10 µl of SensiFAST Probe Hi-ROX Kit (Bioline, UK) Master Mix. After initial denaturation at 95°C (5 min), amplification was performed in 45 cycles of 95°C for 10 s and 60°C for

35 s (acquisition step). All primers and probe used in this study for kobuviruses are listed in Table S1, Supplementary data.

**Complete genome sequencing of enteroviruses.** Primers were designed using VP1 sequences available from this study and reference sequences from GenBank to obtain nearly complete genome of viruses representing EV-G8 and EV-G9 (Supplementary data; Figure S1; Table S2). Nested PCR amplifying regions from about 700 bp to 2.5 kb in length was performed. For each reaction, 6 µl extracted RNA was used for cDNA synthesis in combination with first round PCR using Superscript III one-step RT-PCR System with Platinum Taq High Fidelity (Invitrogen, UK). Then 1 µl of the first-round reaction was used as template for second-round PCR with amplification (using GoTaq DNA polymerase from Promega, UK) conditions as follows: 95°C for 5 min, 30 cycles of denaturation (94°C, 30 s), annealing (50°C, 30 s), and elongation (72°C, 60 s) in a thermal cycler. SequalPrep long PCR kit (Life Technology, UK) was used according to the manufacturer's instruction instead of GoTaq DNA polymerase to amplify fragments longer than 2 kb.

**Sequence analysis.** Sequences were imported into SSE (Simmonds, 2012) for alignment and calculation of sequence divergence values from reference sequences of known enterovirus types. Maximum Likelihood trees were constructed as described in our previous study (Nguyen *et al.*, 2014a) using the MEGA 5.2 software package (Tamura, 2011) with 1000 bootstrap re-samples and best fitting models (Tamura–Nei, gamma distribution for EV VP4/VP2 and VP1, Tamura 3-parameter model, gamma distribution for EV 5'UTR and 3D, general time-reversible gamma distribution with invariant sites for kobuvirus VP1). Nucleotide distance scans and Grouping Scan (Simmonds & Midgley, 2005) implemented in SSE were applied to identify recombination breakpoints. Analysis of phylogenetic groupings was performed by determining association index values (Wang *et al.*, 2001) using the program in SSE with 100 bootstrap replicates.

**Statistical analyses.** Fisher exact test, Chi-squared test and Mann-Whitney test incorporated in Minitab 17 were used to compare data between groups of interest when appropriate as specified.

**Nucleotide sequence accession numbers.** Sequences obtained in this study have been assigned the following GenBank accession numbers: KT265880 to KT266194.

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## Chapter conclusion

In this chapter, the epidemiology, disease association, genetic diversity and recombination of EVs and kobuviruses infecting pigs and boars were examined. Diarrhoea in pigs did not correlate with EV but kobuvirus infection. However, the specific role of kobuviruses remains undetermined. Viruses in positive samples belong to species *Enterovirus G* and *Aichivirus C* which are different from the corresponding viruses reported from humans. Detection of four previously known types and nine new types of *EV-G* indicated the diversity of this species which has not been completely characterised. Meanwhile, analysis of PKV VP1 sequences highlighted the need of serological studies for classification of *Aichivirus C* into (sero)types. Further studies are required for better understanding of their specific disease association and factors that affect their transmission in pigs and boars.

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## Chapter 5. Genetic characterization of enteroviruses in monkeys

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### Introduction

Chapter 3 demonstrated active infection with EVs in monkeys in Cameroon was extremely common with a detection frequency of EV RNA in faecal samples of 96.5%. The genetic diversity of those EVs infecting these primate species will be characterised and compared with EVs previously detected in humans and other NHPs in this chapter. NHPs, in general, are of interest because their close genetic relationship with humans may facilitate the exchange of zoonotic pathogens between humans and NHPs. In fact, this has been shown by detection of a large number of human EV serotypes in NHPs with unknown transfer direction (Harvala *et al.*, 2011b, Oberste *et al.*, 2013a, Oberste *et al.*, 2013b, Harvala *et al.*, 2014, Sadeuh-Mba *et al.*, 2014). NHPs may thus act as a potential source of new human epidemics (Harvala *et al.*, 2011b). The current work is a larger scale continuation of previous studies of EV infection in African primates (Harvala *et al.*, 2011b, Harvala *et al.*, 2012, Harvala *et al.*, 2014) and is in line with the remit of this thesis looking for zoonotic sources of human infections.

### Picornavirus taxonomy and species definition

Picornaviruses have now been classified into species and genera on the basis of their phylogenetic relationships, usually with supporting information such as genome organization, biological properties and host range (<http://www.ictvonline.org/codeOf-VirusClassification.asp>). The ultimate aim of taxonomy is to produce a hierarchical system of species, genus, family and order that reconstructs phylogeny and reflects the degree of biological similarity. The classification of viruses, however, has particular difficulties due to the rapidity with which they evolve (Knowles *et al.*, 2010). The continual discovery of candidates for novel picornaviruses makes the classification of this virus family, which is still in flux, more complicated (Knowles *et al.*, 2012).

Although species is a basic unit of taxonomy, its definition was only considered in preparation for the publication of the Seventh ICTV Report in 2000 (King *et al.*, 2000).

That was when the ICTV began to grapple with the problem of defining it: how to define species in rapidly mutating organisms? Previously, the designation of picornavirus species had been the prerogative of the Picornavirus Study Group who had referred each serotype as a separate “species”. Under the modern definition, in contrast, “a picornavirus species is a class of phylogenetically related serotypes or strains which would normally be expected to share (i) a limited range of hosts and cellular receptors, (ii) a significant degree of compatibility in proteolytic processing, replication, encapsidation and genetic recombination, and (iii) essentially identical genome maps” (Knowles *et al.*, 2010).

The core concept behind the contemporary virus taxon is exclusivity which prevents one taxon from containing another at the same level. No species, for example, may contain another species (this is one of the reasons why “*Poliovirus*” lost its species status). The availability of precise measures of genetic distance has made the current emphasis on the phylogeny possible. Previous picornavirus classifications used criteria such as pathogenicity observed in experimental infection of animals or cultured cells, but these have been found to often correlate poorly with their underlying genetic relationships. However, some relics of this old nomenclature are still in use, such as coxsackievirus A24 a member of species *EV-C* while other coxsackieviruses are in species *EV-A*, *EV-B* (Knowles *et al.*, 2010).

### **Species demarcation criteria in the *Enterovirus* genus**

The criteria for classification into species of members in the *Enterovirus* genus, as well as other picornavirus genera, have been re-examined in each ICTV report. The latest report (Knowles *et al.*, 2012) stated that in practice, members of a species in this genus have a genome base composition (G + C) which varies by no more than 2.5%, and share:

- > 70% aa identity in the polyprotein
- > 60% aa identity in P1
- > 70% aa identity in the non-structural proteins 2C + 3CD
- a limited range of host cell receptors
- a limited natural host range

- a significant degree of compatibility in proteolytic processing, replication, encapsidation and genetic recombination.

### **Role of recombination in maintenance of picornavirus species**

Recombination plays an important role in the microevolution of such viruses with high mutation rates as picornaviruses. On a short time and space scale, it is viewed as a mechanism for removal of deleterious mutations whereby recreating a functional genome from impaired ones. It is also a means to increase the genomic diversity of picornaviruses (Lukashev, 2010) as demonstrated by frequent emergence of new picornavirus recombinant forms (Cabrerizo *et al.*, 2014; Gaunt *et al.*, 2015; McWilliam Leitch *et al.*, 2009; McWilliam Leitch *et al.*, 2012). Over the long term, recombination in most picornaviruses functions as a driving force which helps preserve the integrity of species by frequent shuffling diverging genome fragments. This hypothesis implies that viruses of the same species must undergo recombination with each other over a limited period of time (probably on a time scale of decades), and therefore restrict their sequence divergences below a certain threshold. Such degree of diversity is thought to be biologically compatible enough to allow emergence of viable recombinants. Without recombination, a high mutation rate would lead to the formation of a new species or severe deterioration of the virus sequence (Lukashev, 2010).

### **Impact of recombination on classification of EVs**

In general biology, a species is defined as “a group of organisms able to interbreed and produce fertile offspring” (Tisdall, 1981). It can be inferred from this definition that all organisms of a species have a common gene pool and restricted divergence. Recombination in EVs can be viewed as an analogue of such reproductive strategy in higher organisms (Lukashev, 2010). On analysis of *EV-B* sequences, Lukashev *et al.* (2003) noticed that recombination strictly occurred within this species. Consequently, the possibility of natural recombination was proposed as an additional species criterion in EVs (Lukashev *et al.*, 2003).

Nonetheless, some complicated issues relating to the inclusion of this criterion arose on a more thorough examination of genus *EV*. Due to closely relatedness and identity in the genome sequences, a number of simian EVs were classified in species *EV-A* with human EVs. However, these two EV groups do not recombine with each other and would eventually evolve into different species. Similarly, SVDV no longer recombines with other *EV-B* viruses after the host switch from humans to pigs (Zhang *et al.*, 1999) and would most likely diverge from *EV-B* to form a new species within decades (Lukashev, 2010). As another example, CVA1, CVA19 and CVA22 form a monophyletic clade throughout the genome without evidence of recombination with other serotypes in species *EV-C* (Brown *et al.*, 2003). Unlike other human EVs, these three *EV-C* serotypes are unable to grow in cell culture, probably due to different receptor usage. These viruses may also be on the process of speciation (Lukashev, 2010). On the other hand, recombination between EV species (e.g. *Rhinovirus A* and *C*) also occurs although at a rarer frequency and only in highly conserved regions such as the 5'UTR (Simmonds, 2010).

The genetic diversity and recombination of EVs in monkeys were investigated in this chapter and published in 2014 (Nguyen *et al.*, 2014b).

## **High Rates of Infection with Novel Enterovirus Variants in Wild Populations of Mandrills and Other Old World Monkey Species**

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## High Rates of Infection with Novel Enterovirus Variants in Wild Populations of Mandrills and Other Old World Monkey Species

### ABSTRACT

Enteroviruses (EVs) are a genetically and antigenically diverse group of viruses infecting humans. A mostly distinct set of EV variants have additionally been documented to infect wild apes and several, primarily captive, Old World monkey (OWM) species. To investigate the prevalence and genetic characteristics of EVs infecting OWMs in the wild, fecal samples from mandrills (*Mandrillus sphinx*) and other species collected in remote regions of southern Cameroon were screened for EV RNA. Remarkably high rates of EV positivity were detected in *M. sphinx* (100 of 102 screened), *Cercocebus torquatus* (7/7), and *Cercopithecus cephus* (2/4), with high viral loads indicative of active infection. Genetic characterization in VP4/VP2 and VP1 regions allowed EV variants to be assigned to simian species H (EV-H) and EV-J (including one or more new types), while seven matched simian EV-B variants, SA5 and EV110 (chimpanzee). Sequences from the remaining 70 formed a new genetic group distinct in VP4/2 and VP1 region from all currently recognized human or simian EV species. Complete genome sequences were obtained from three to determine their species assignment. In common with *EV-J* and the *EV-A* A13 isolate, new group sequences were chimeric, being most closely related to *EV-A* in capsid genes and to *EV-B* in the nonstructural gene region. Further recombination events created different groupings in 5' and 3' untranslated regions. While clearly a distinct EV group, the hybrid nature of new variants prevented their unambiguous classification as either members of a new species or as divergent members of *EV-A* using current International Committee on Taxonomy of Viruses (ICTV) assignment criteria.

### IMPORTANCE

This study is the first large-scale investigation of the frequency of infection and diversity of enteroviruses (EVs) infecting monkeys (primarily mandrills) in the wild. Our findings demonstrate extremely high frequencies of active infection (95%) among



mandrills and other Old World monkey species inhabiting remote regions of Cameroon without human contact. EV variants detected were distinct from those infecting human populations, comprising members of enterovirus species B, J, and H and a large novel group of viruses most closely related to species A in the P1 region. The viral sequences obtained contribute substantially to our growing understanding of the genetic diversity of EVs and the existence of interspecies chimerism that characterizes the novel variants in the current study, as well as in previously characterized species A and J viruses infecting monkeys. The latter findings will contribute to future development of consensus criteria for species assignments in enteroviruses and other picornavirus genera.

## INTRODUCTION

Enteroviruses (EVs), members of the genus *Enterovirus* within the family *Picornaviridae*, are notable for their genetic and antigenic diversity with more than 300 types identified thus far. EVs are currently classified into nine species (labelled alphabetically as *EV-A* to *EV-H* and *EV-J*). This subdivision of the *Enterovirus* genus is based on degrees of sequence divergence, host range, similarities in replication, and a generally observed restriction of recombination between members of the same species (1). The enteroviruses infecting humans comprise *EV-A* to *-D*, while the remaining five contain viruses infecting primarily cattle (*EV-E* and *EV-F*; both termed bovine enteroviruses), pigs (*EV-G*; porcine enteroviruses) and monkeys (*EV-H* and *EV-J*; both simian enteroviruses) (1). However, EV variants classified as species A, B, and D have also been detected in several monkey and ape species (2 – 13).

The first simian EVs were discovered in the 1950s and 1960s in primate cell cultures or from primate tissue specimens used in biomedical research. These EVs were obtained from Old World monkey (OWM) species *Macaca mulatta* (rhesus macaque), *Macaca fascicularis* (cynomolgus monkey), *Chlorocebus aethiops* (African vervet monkey), and *Papio cynocephalus* (baboon) (2 – 7, 14). Subsequent genetic characterization indicated that some isolates were similar to human viruses (A13, SV19, SV43, and SV46 in species A; SA5 in *EV-B*), while others were genetically distinct (9 – 11) and now classified into two separate species (e.g., SV4 and SV28 in

EV-H and SV6 in EV-J). More recent genetic characterization of EVs infecting captive primates (mostly rhesus macaques [83%]) in the Yerkes National Primate Center in the United States and at the Dhaka Zoo in Bangladesh identified four further simian EV types (EV92 in species A; EV103, EV112, and EV115 in species J) (13, 15), whereas no simian EVs were detected in synanthropic (e.g., pet) monkeys (12).

To investigate whether EVs also circulated in apes, we recently performed large-scale screening of fecal samples collected from wild populations of chimpanzees (*Pan troglodytes*) and gorillas (*Gorilla gorilla*) primarily in Cameroon. From these fecal samples, four EV types (EV76, EV89, and EV119 in species A; EV111 and EV120 in species D) and a novel species B variant (EV110) were identified (16, 17). However, as previously discussed (17), whether these represent endogenous infections in these species or have been acquired from contact with humans or monkeys remains unclear; remaining wild-living chimpanzee and gorilla populations are small and highly fragmented. The overall detection frequencies were however moderate (9 to 11%), and the populations sampled had minimal contact with human populations in sample collection areas.

To understand more about the natural circulation of EVs in wild primate populations, we analyzed a large collection of samples from mandrills (*Mandrillus sphinx*) and smaller numbers of other OWMs in isolated areas of Cameroon. Mandrills were selected because populations and groupings are large and more likely to support endogenous populations of EVs than apes. Genetic characterization of the extensive numbers of variants detected demonstrated the broad diversity of species and types circulating in these populations in the wild. A wider phylogenetic comparison of complete genome sequences of OWM, ape, and human EVs was performed to determine their species relationships to other simian and human EVs.

## MATERIALS AND METHODS

**Samples.** A total of 102 mandrill (*Mandrillus sphinx*), 7 red-capped mangabey (*Cercocebus torquatus*), and 4 moustached monkey (*Cercopithecus cephus*) stool samples were included in this study (Table 1; see Tables S1 and S2 in the supplemental material). Samples were collected from natural habitat areas of mandrills - remote and

sparsely populated forested southern areas of Cameroon with minimal human contact (18). Samples were preserved in RNAlater (Ambion, Austin, TX), stored at room temperature at base camps for a maximum of 3 weeks, and subsequently transported to a central laboratory in Yaoundé, Cameroon, for storage at -20°C/-80°C. For all fecal samples, the species origin was also determined by mitochondrial DNA (mtDNA) analysis as described previously (19). Fecal DNA was extracted using the QIAamp stool DNA minikit (Qiagen, Valencia, CA).

**Sample extraction and amplification.** RNA was extracted from fecal samples as previously described (20) and tested for enterovirus (EV) by one-step reverse transcriptase (RT)-PCR assay targeting the 5' untranslated regions (5'UTRs) (see Table S3A in the supplemental material) (21). To estimate viral loads in EV-positive samples, samples were retested by real-time PCR calibrated using defined copy numbers of EV RNA transcripts (22). Additional primer sets were used to obtain sequence data from VP4 and VP1 region as described (Table S3B) (16, 17). The amplification of VP1 required two sets of primers, which amplify overlapping regions from each species, and additional primers specific for the species B variant SA5 (as indicated in the primer name). For each PCR, 6 µl extracted RNA was amplified by a combined RT and first-round PCR using Superscript III one-step RT-PCR system (Invitrogen, United Kingdom), and 1 µl of the first-round reaction was used for the nested PCR with second-round primers. PCR amplification included 30 cycles of denaturation (94°C, 20 s), annealing (50°C, 18 s), and elongation (72°C, 90 s) in a thermal cycler.

**Whole-genome sequencing.** The whole genomes of EV variants representing three of the predominant types infecting mandrills were sequenced (samples GR2815, CPML8109, and CPML3961). Primers were designed using sequence data in the VP4 and VP1 regions available from the current study and sequences of viruses in species J. One microliter of cDNA synthesized by using random hexamer primers and Superscript III reverse transcriptase (Invitrogen, United Kingdom) was used as the template for nested PCR. The amplification conditions were the same as those of the second-round PCR mentioned above. Assemblies were created from sets of approximately 1.5kb sequence fragments in which the regions of overlap were

identical between fragments; this prevented generation of possible hybrid viruses from samples from monkeys that may be coinfecting with two or more EV variants.

**Direct sequencing of PCR products.** Positive second-round PCR products were sequenced in both directions using the inner sense and inner antisense primers used in the second round of amplification, using BigDye Terminator v3.1 (Applied Biosystems). Nucleotide sequences were assembled, annotated, and aligned using the SSE sequence editor version 1.1 (23).

**Genetic analysis of EV sequences.** Sequence comparisons were made between the Old World monkey-derived sequences obtained in the current study with all available sequences from VP1, VP4, 3Dpol, 5'UTR, and 3'UTR sequences of variants previously reported for other nonhuman primates. All data sets additionally included a single representative sequence derived from complete genome sequences of each human EV type. Calculation of pairwise distances between sequences and divergence scans were performed using SSE. Phylogenetic trees constructed using maximum likelihood methods as implemented in the MEGA 6 software package (24). The optimum maximum likelihood model (lowest Bayesian information criterion score and typically greatest maximum likelihood value) for each sequence data set was first determined and used for phylogenetic reconstruction. For each genome region (5'UTR, VP4/VP2, VP1, and 3CD), this was general time reversible (GTR) with a gamma ( $\gamma$ ) distribution (5 rates) and invariant sites (I) (GTR plus  $\gamma$  plus I). Phylogenetic analysis of each data set used 100 bootstrap resamplings to determine the robustness of grouping.

**Nucleotide sequence accession numbers.** Sequences obtained in the current study have been submitted to GenBank and have been assigned the accession numbers KJ420625 to KJ420749.

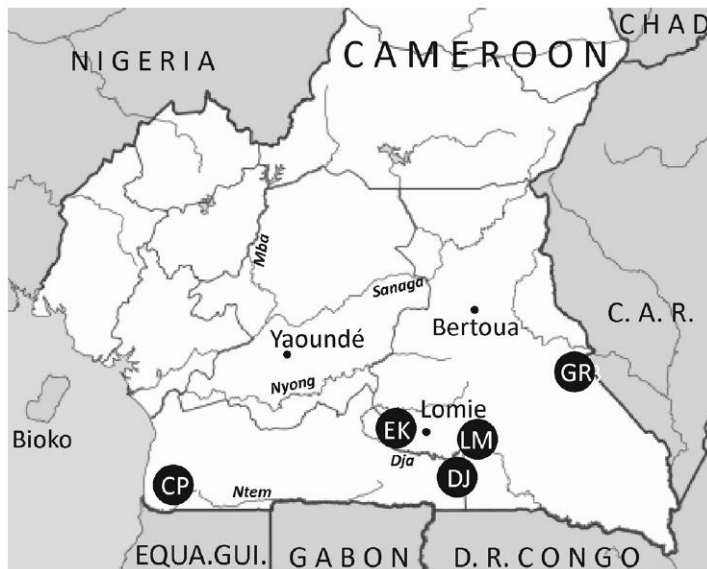
## RESULTS

**EV RNA detection frequencies and viral loads.** RNA was extracted from 102 mandrill, 7 red-capped mangabey, and 4 moustached monkey fecal samples and screened for EV RNA sequences using primers targeting a region of the 5' untranslated

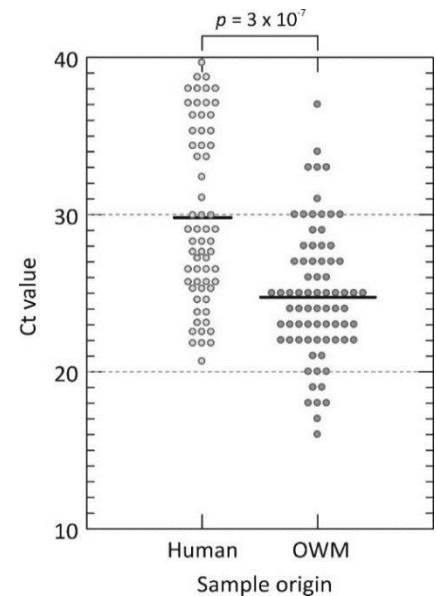
region (5'UTR). From these fecal samples, 100 of 102 mandrill samples (98%), all red-capped mangabey samples, and two moustached monkey samples were positive. Sampling sites were located throughout southern Cameroon (Figure 1; see Tables S1 and S2 in the supplemental material). The mean viral loads of EV in mandrill fecal samples were about 10-fold higher than those from a representative set of human samples tested in the same assay (25) (Figure 2), with mean threshold cycle (Ct) values of 27.5 compared to 29.8 in human samples ( $p = 0.001$  by the Kruskal-Wallis test).

**Table 1.** Detection frequencies in study samples using primers from different genome regions

Species	Detection frequency (no. of positive samples/total no. of samples tested)		
	5'UTR	VP4/2	VP1
<i>Mandrillus sphinx</i>	100/102	88/100	39/88
<i>Cercocebus torquatus</i>	7/7	3/7	0/3
<i>Cercopithecus cephus</i>	2/4	2/2	0/2



**Figure 1.** Collection sites in Cameroon of samples used in the current study. The collection sites in Cameroon were abbreviated as follows: CP, Campo, locations Oveng, Melen, and Grothes (CPOV, CPLM, and CPGR, respectively); DJ, Djoum; EK, Ekom; LM, Lomieu; GR, Gribi. C. A. R., Central African Republic, EQUA. Gui., Equatorial Guinea, D. R. Congo, Democratic Republic of the Congo.



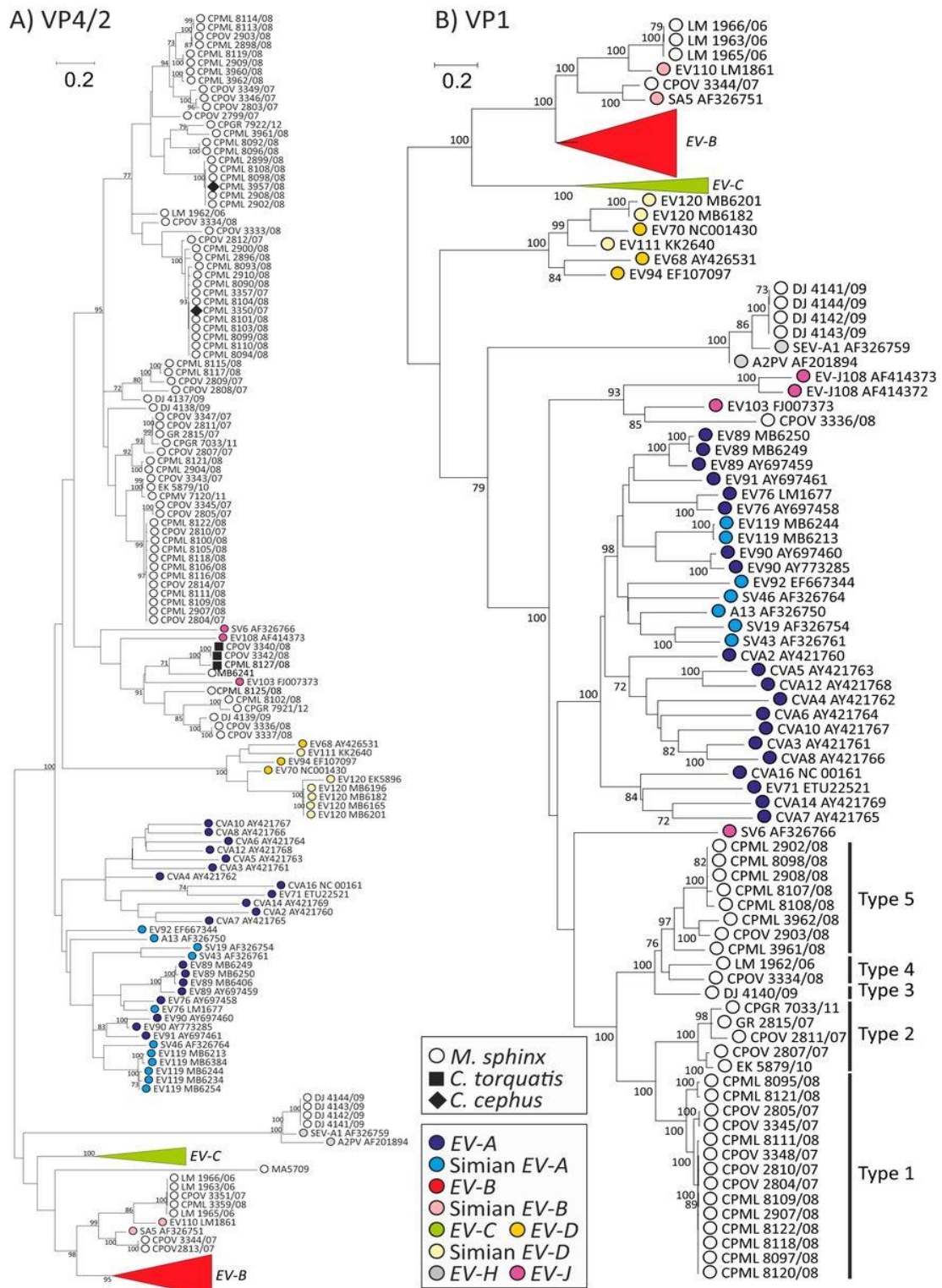
**Figure 2.** Comparison of Ct values of EV RNA detected by real-time PCR in faecal samples collected from mandrills with those in human samples. Each symbol represents the value for one fecal sample. Bars show mean values. Statistical significance was performed using Kruskal-Wallis nonparametric test (above graph).

**EV type assignments.** To genetically characterize EV variants infecting these OWMs, sequences were amplified from the VP4/2 region. A total of 88 of 100 samples from mandrills were positive, from which 84 were successfully sequenced, as were 3 of 7 mangabey samples and two *Cercopithecus* (moustached monkey) samples (Figure 3A). For the purposes of further type identification and new type assignments, sequences were amplified in the VP1 region from 39 mandrill samples (Figure 3B).

The viruses identified fell into three previously identified enterovirus species. Four could be assigned to species H that includes the simian viruses A-2 plaque virus and SEV-A (isolated from the OWM species *Macaca mulatta*). VP1 sequences showed 14.5 to 15.5% nucleotide sequence divergence from previously characterized simian variants in this species, suggesting that they should be classified as EV-H1. However, the four samples containing species H viruses were collected from the same site on the same day; due to the nature of sampling from the jungle floor and their high sequence identity, it was likely that they originated from the same group or possibly even the same mandrill.

Other variants grouped with the simian enteroviruses SA5 and EV-B110 within species B. Five of these variants could be assigned as EV110 (20.6% to 20.8% divergence in VP1), a recently described EV type detected in a chimpanzee from Cameroon (17), while a further two could be assigned as SA5 (15.5% divergence in VP1) that was originally isolated from a vervet monkey (*Cercopithecus aethiops* [4]).

VP4/2 sequences of a further 10 variants clustered in species J (7 derived from mandrills and 3 derived from mangabeys), a species into which the simian viruses SV6 (*M. mulatta*), EV103 (*Macaca nemestrina*), and EV108 (*Papio cynocephalus*) are currently assigned. From these, a VP1 sequence was obtained from sample CPOV3336, which was > 25% divergent from assigned types within this species. It is likely, however, that several other variants correspond to further *EV-J* types based on their sequence divergence in VP4/2.



**Figure 3.** Maximum likelihood analysis (GTR plus  $\gamma$  plus *I* model) of VP4/partial VP2 region (A) and whole VP1 (positions 743 to 1168 and 2477 to 3376, respectively, numbered using the PV3 reference sequence) (B). Sequences amplified from study samples are shown as white and solid black symbols, while those previously assigned within EV species A to D, J, and H are color coded according to the key (human EV-B and EV-C sequences were monophyletic and have been collapsed to clarify presentation of simian sequences). Bootstrap resampling of maximum likelihood (ML) trees was performed to indicate robustness of grouping (values of  $\geq 70\%$  shown).

The remainder of viruses form a monophyletic group in both VP4/2 (67 mandrill sequences and 2 *Cercopithecus* sequences) and VP1 (30 mandrill sequences). In the VP1 region, sequences showed substantial nucleotide sequence divergence from each other and supported their assignment into 5 types (Figure 3B) based on > 25% pairwise nucleotide distances. How these should be named is dependent on their final species assignment, and this remains uncertain based on phylogenetic analysis of VP1 and VP4/2 region sequences.

**Species assignment.** To clarify the species assignment of the new, predominantly mandrill, group and investigate its relationships with other simian and human EVs, complete genome sequences were obtained from variants representing 3 of the 5 putative types (samples GR2815, CPML8109, and CPML3961) within the group. This allowed current molecular demarcation criteria of amino acid sequence identity in the polyprotein, P1, and combined nonstructural gene region 2C plus 3CD (1) to be used for species assignments (Table 2).

**Table 2.** P1, 3CD, and whole-genome amino acid sequence distances between mandrill variants and other species

Species and subgroup	Amino acid sequence distance <sup>a</sup>		
	Whole genome	P1	2C + 3CD
<i>EV-J</i>	<b>0.264</b>	<b>0.384</b>	<b>0.1682</b>
<i>EV-C</i>	0.403	0.514	<b>0.2934</b>
<i>EV-A</i>			
All	0.351	<b>0.3714</b>	0.3334
A1	0.3595	<b>0.379</b>	0.341
A2	0.3235	<b>0.346</b>	0.3074
<i>EV-B</i>			
All	<b>0.2981</b>	0.5107	<b>0.158</b>
Human	<b>0.2996</b>	0.511	<b>0.1596</b>
SA5	<b>0.2489</b>	0.493	<b>0.0613</b>
<i>EV-C</i>	0.4034	0.514	<b>0.2934</b>
<i>EV-D</i>	0.375	0.463	<b>0.2853</b>
<i>EV-H</i>	0.3905	0.465	0.3085

<sup>a</sup> Amino acid sequence distances below species assignment thresholds are shown in boldface type (complete coding region, 30%; P1, 40%; 2C plus 3CD, 30%).

As suspected from the differences in phylogenetic groupings between the new variants and other EV species (Figure 3B), amino acid sequence distances between this group



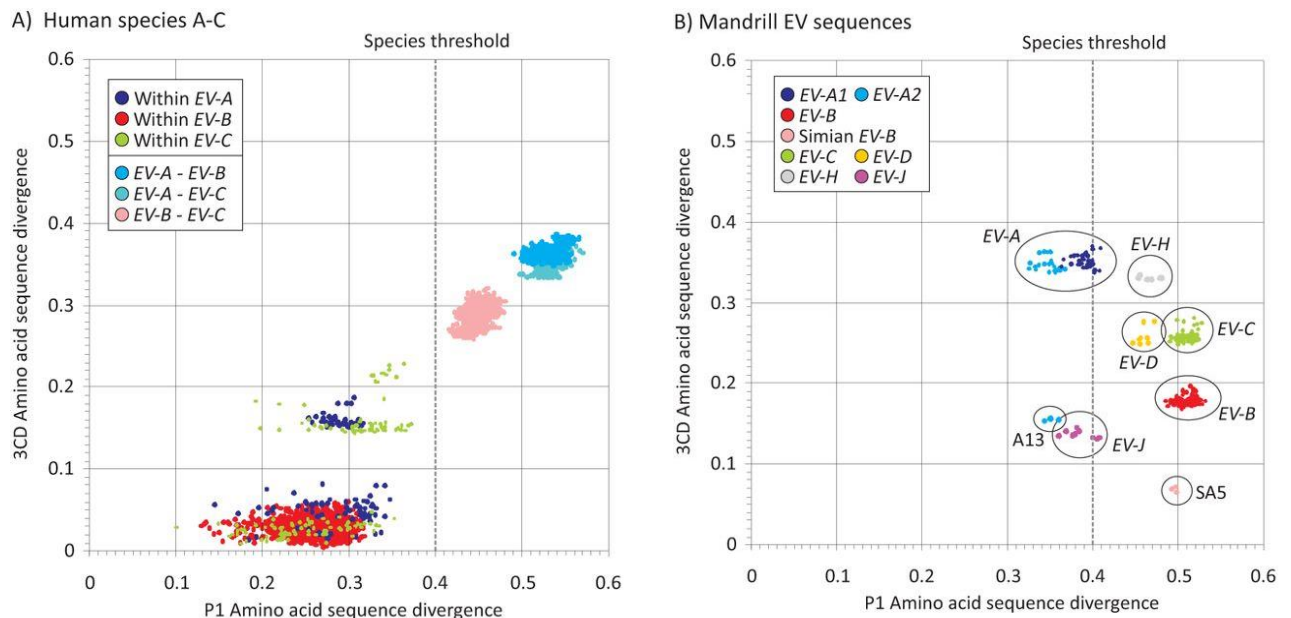
and species J and A viruses in P1 were close to but consistently below the 40% previously proposed species assignment threshold (38.4% and 37.9%, respectively; Table 2). Furthermore, if species A viruses were divided into the two subclades apparent in VP1 (one comprising exclusively human serotypes, termed A1), and the other simian and more recently discovered human-derived types EV76, -89, -90, and -91 (termed A2) (26), then further problematic relationships arise. The new mandrill variants showed only 34.5% divergence from the predominantly simian A2 group. A2 variants themselves show only 38.9% divergence from species J.

Sequence divergence in the combined 2C plus 3CD region did not resolve the species status of the mandrill sequences and indeed revealed further inconsistent sequence relationships with other variants. With a proposed 30% amino acid divergence threshold, new variants showed remarkably low divergence from both *EV-J* (16.8%) and *EV-B* (15.8%). Furthermore, mandrill variants and the simian species B isolate SA5 showed only 6.1% divergence. These interrelationships are mirrored by the greater than expected sequence similarity of *EV-J* to *EV-B* (21.8% divergence). These inconsistencies in sequence relationships in different genome regions were not resolved by comparison of whole polyprotein sequences, where mandrill variants showed below-species divergence thresholds (set at 30%) of 26.4% with *EV-J* and 29.8% with *EV-B*.

Information for the mandrill variants that contribute to the other species assignment criteria (1) was largely unresolved or ambiguous, including host range (infects at least two different OWM species), host cell receptors (unknown), and compatibility in proteolytic processing, replication, encapsidation, and genetic recombination (unknown). However, the three variants show a narrowly constrained range of GC compositions (44.3% to 44.6%; threshold range of 2.5%).

**Chimerism in EV genomes.** It is possible that the difficulty associated with a species designation of the new variants using conventional criteria had originated from their possession of chimeric genomes comprising sequences originating from different EV species. To investigate this, relationships between inter- and intraspecies distances in P1 and 3CD were compared (Figure 4). For the presumed nonchimeric human species

A, B, and C sequences, sequence distances in P1 were approximately proportional to those in 3CD (Figure 4A). Comparison of variants assigned to different species invariably showed distances of  $> 40\%$  in P1 and  $> 25\%$  in 3CD (including *EV-D*; not shown). The main exception to the linear relationship between distances was the frequently suppressed variability of 3CD sequences between different types within a species. As documented elsewhere, this originates through promiscuous intertype recombination in the non-structural gene regions, where different types within a species frequently share a pool of less-divergent NS gene region sequences (27 – 30). Species A and C, however, contain subgroups with more divergent NS gene region sequences (15% to 23%; Figure 4A). In species A, these represent distances between subgroups corresponding to the previously described EV-A1 (all human) and -A2 groups (EV76, -89, -90, and -91) (26). In species C, they originate from the previously identified more-divergent variants CAV1, CAV19, and CAV22 (31) and EV104 (32).



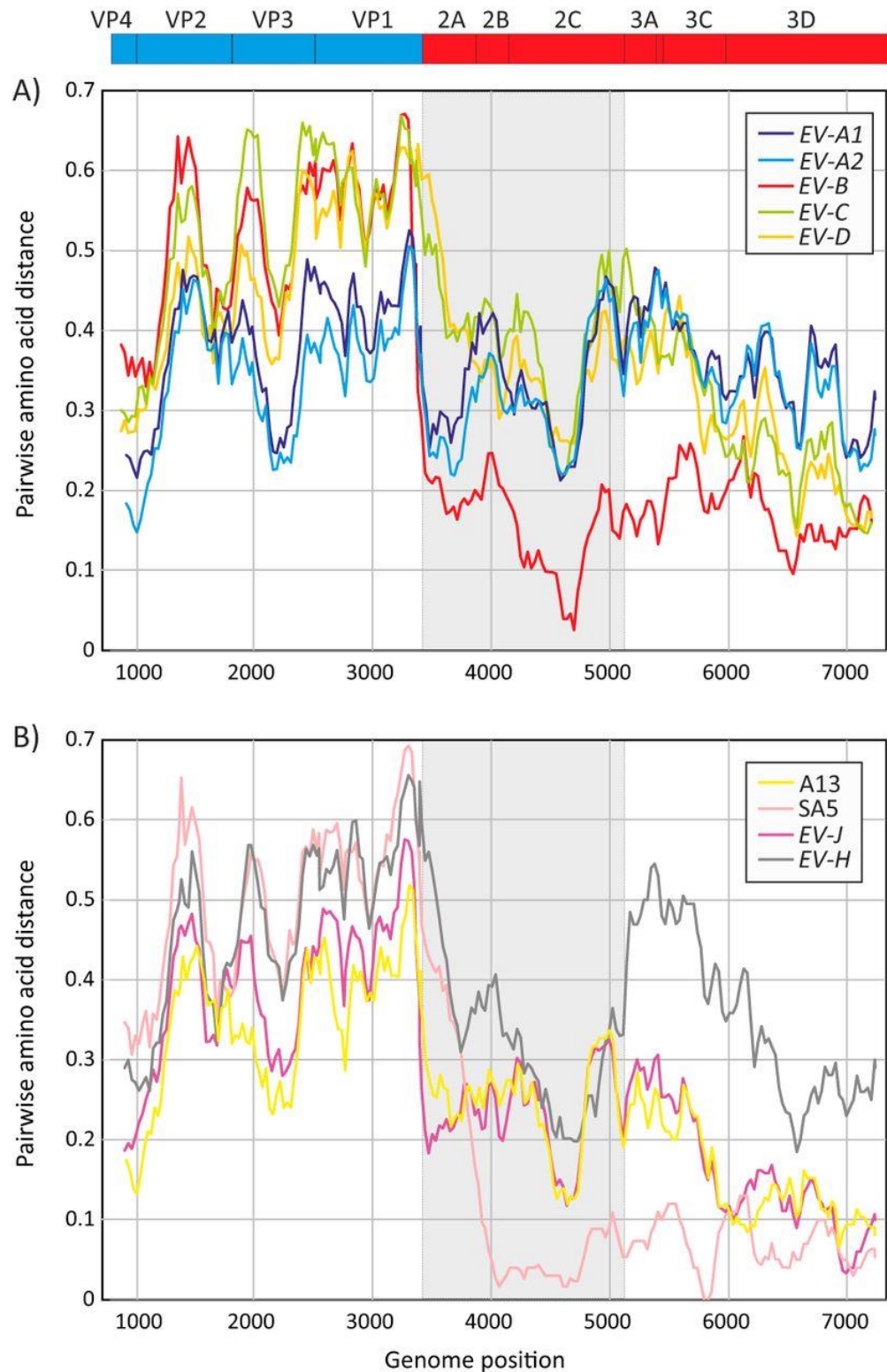
**Figure 4.** (A) Distributions of pairwise amino acid distances in P1 (positions 743 to 3376) and 3CD regions (positions 5429 to 7360) between representative human-derived sequences within *EV-A*, *EV-B*, and *EV-C*. Color coding has been used to identify within- and between-species sequences distances for each. The 40% amino acid distance threshold for species assignments using P1 is shown as vertical gray broken line. (B) Pairwise distances between three representative mandrill sequences with EV species A to D, J, and H.

An equivalent comparison of sequences from the mandrill group with other species showed combinations of P1 and 3CD regions that were markedly inconsistent with

relationships between human-derived viruses (Figure 4B). Only comparisons with *EV-C*, *EV-D*, and *EV-H* produced pairwise distances consistently above 40% (P1) and 25% (3CD) indicative of unambiguous separate species status. In contrast, most sequence comparisons of mandrill viruses with species B and J variants were in the intraspecies range in 3CD, with divergence values of 15% to 23% that were comparable to those between subgroups of *EV-A* and *EV-C* (Figure 4A). Furthermore, mandrill sequences differed by only 6.8 to 7.3% from SA5, which are typical of within-recombination pool distances in human *EV-A – EV-C* (Figure 4A). Mandrill viruses showed the opposite relationship with *EV-A* with P1 distances of < 40% (intraspecies range) but > 25% in 3CD (interspecies).

Comparison of sequence distances in P1 and 3CD sequences therefore provided evidence for chimerism (from previous interspecies recombination) in the mandrill sequences between these regions. To identify sites where sequence exchanges occurred, divergence plots were constructed between mandrill sequences and the nonrecombinant human species A to D (Figure 5A) and with simian viruses A13, SA5, and species H and J (Figure 5B). As expected from their consistent sequence relationships in VP1 and 3CD, divergence between mandrill viruses and human species A, C, and D and simian species H were comparable across the EV coding region (Figure 5A and B). Comparison with species B, however, identified highly divergent capsid sequences, and an abrupt decline into interspecies divergence levels at the P1/P2 (VP1/2A) boundary. A different breakpoint was observed with the simian *EV-B* sequence SA5 (Figure 5B), situated approximately 400 bases downstream from P1/P2 within 2A.

Comparison of mandrill viruses with simian variants *EV-J* and A13 provided evidence for further chimerism, with sequences from the P2 region (shaded area in [Figure 5B](#)) close to the within species range (20% to 35%) and comparable to distances from *EV-H*. However, in P3, distances were substantially lower in the within-species range. These observations provide evidence for further genetic exchanges in the evolution of mandrill viruses, one or more of which are likely ancestral to their split from *EV-J*.

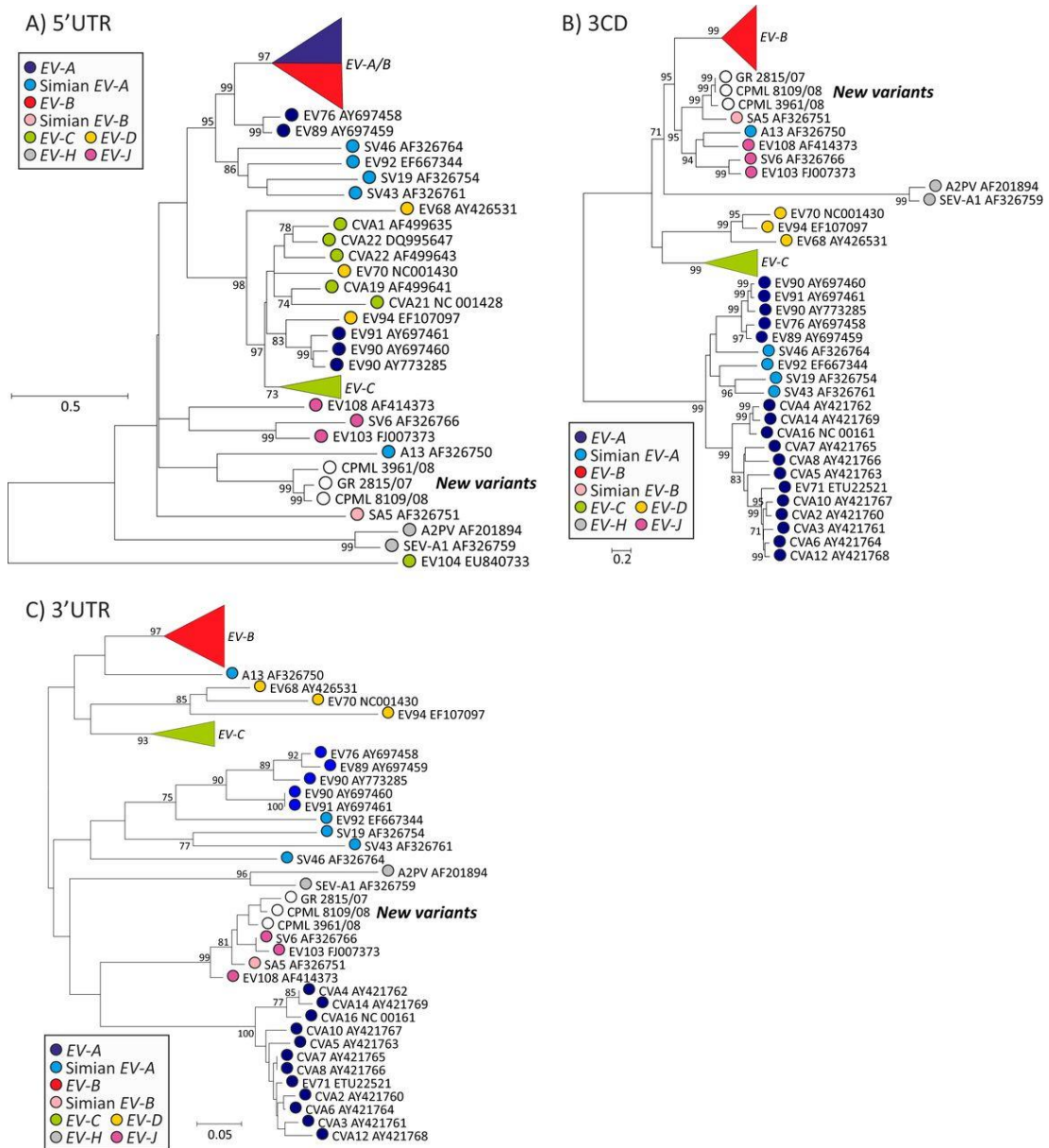


**Figure 5.** Amino acid sequence divergence between mandrill sequences and A1 and A2 subgroups in *EV-A*, human variants in species B to D (A) and simian viruses in species A, B, J, and H (B). The values on the y axes show mean pairwise distances between 300-base windows across the coding region, in increments of 30 bases between fragments. To show gene boundaries, the P2 region has been shaded gray, and a representation of gene positions in the EV polyprotein (drawn to scale) is shown above the graphs.

**Genetic exchange of 5'- and 3'UTR sequences.** To investigate the genetic relatedness of noncoding regions at the ends of the EV genomes, phylogenies were constructed from whole 5'UTR and 3'UTR and compared to those of 3CD (Figure 6) and previously constructed trees from structural gene regions VP4/2 and VP1 (Figure 3). Sequence relationships in both noncoding regions were distinct from those of coding regions and from each other, with consistent separate groupings of simian-derived viruses from human variants and in the case of the 5'UTR, the existence of individual clades containing human variants of more than one species, *EV-A* and *-B* in one and *EV-C*, *-D*, and *-A* in another (33). In the 3'UTR, mandrill viruses, *EV-J* and SA5 grouped together as observed in 3CD, but this group excluded A13 and human species B. The other striking phylogeny difference was the complete separation of human A2 viruses from A1 that were in turn distinct from simian A2 viruses. Although the 3'UTR was short and tree construction was intrinsically less robust than for other genome regions, these observations provide evidence for genetic exchanges, additional to those between nonstructural gene regions and distinct from those occurring in the 5'UTR.

## DISCUSSION

This study is the first large-scale investigation of infection frequencies and genetic characterization of enteroviruses infecting Old World monkeys in the wild. The extraordinarily high prevalence of active infection and genetic diversity of EVs found provides evidence for the extensive circulation of these viruses in wild primate populations. The sampling strategy that concentrated collection on remote areas with no or minimal human contact and the reclusive nature of the species (34) helped rule out human sources of infection in monkeys, as did the finding of virus variants that were consistently genetically distinct from human enteroviruses. Primate sampling of faeces has been commonly used to investigate infection frequencies of simian immunodeficiency virus (18), and collection methods used for the current study were specifically designed to avoid environmental contamination. Furthermore, samples showed generally high viral loads (mean Ct values of 27.5), indicating substantially higher viral loads than typically found in human samples (Figure 2) and demonstrating active infections in the sampled populations.



**Figure 6.** Phylogenetic analysis of parts of 5'UTR (A), 3CD (B), and 3'UTR (C). (A) 5'UTR, positions 1 to 742; (B) 3CD, positions 5429 to 7366; (C) 3'UTR, positions 7377 to 7431. Bootstrapped ML trees were constructed as described for in the legend to Figure 3 with the exception of the 3'UTR where its short sequence length (55 bases) precluded meaningful model fitting. The tree is therefore shown as a phenogram by neighbor joining of uncorrected nucleotide *p*-distances.

The nature of the sampling, however, prevented more-focused investigations of EV persistence and disease associations in mandrills or other monkey species. This limitation additionally prevented clear attribution of samples to individual (different) monkeys. This sampling bias is exemplified by the four species H variants that originated from samples collected on the same day from the same site. It is likely that they originated from members of the same group (and thus are epidemiologically



linked infections) or conceivably from the same mandrill. The development of genetic fingerprinting for mandrills and other monkey species, as used in ape samples, would be needed to investigate this further. Nevertheless, the vast majority of EV variants characterized in the study were genetically distinct from each other and originated from widespread sampling (throughout southern Cameroon) over a prolonged period (2008 to 2012). In the main, therefore, they represent independent infections.

Infection frequencies of greater than 95% in mandrills were substantially higher than the 10% detected in apes (chimpanzees and gorillas) from similar sampling areas (16). Although little is known about possible differences in susceptibility or persistence of EV infections in OWMs, apes, and humans, a major factor likely contributing to the circulation of EVs in mandrills is the large and highly interactive populations of mandrills in the wild, which typically live in hordes of between 500 and 1,000 (34). This population size and their interactions with other groups of mandrills and other OWMs may create the necessary conditions to permit ongoing transmission of acute virus infections over long periods. Indeed, even under captive conditions, infections with EV variants found previously in monkeys were frequently detected even in small isolated OWM populations in a primate center and a zoo (13, 15), while only human serotypes were detected among nonhuman primates living in human environments (12). Our findings support the broader hypothesis that EV infections are prevalent in at least one OWM species and continue to circulate in the absence of human contact or intervention.

The EV types detected in the current study fell into species H and J and a large, new group of currently undefined taxonomic status within the *Enterovirus* genus. No variants that corresponded to types or species found in humans were detected, confirming the absence of human sources of infection in mandrills and other sampled OWM species. Among the 89 EV variants characterized by VP4/2 sequencing, several could be classified as members of species H type 1 variants previously detected in a simian organ cultures from an Asian ape, *M. mulatta* (SV2, SV4, and SV28) (6) but also from human subjects with hepatitis in the early 1960s (35, 36). Others grouped in species J, a group now listed as containing six assigned types (but with sequences available from only three [SV6, EV103, and EV108]), to which the sequence of

CPOV\_3336 may contribute an additional type based on its divergent VP1 sequence. Although most *EV-J* variants have been identified in African OWMs, the originally described SV6 isolate originated in an Asian macaque (6). Finally, several mandrill-derived variants could be classified as members of the simian subgroup of species B, one corresponding to SA5 and the other to EV110, previously identified in chimpanzees from the same geographical area as the mandrill samples (Lomie [LM] in Figure 1) (17).

Genetic characterization of the remaining, predominantly mandrill-derived EVs was performed by extensive sequence analysis of VP4/2 and complete VP1 sequences. From the latter, the existence of at least 5 types differing by > 25% in nucleotide sequence could be inferred, although their definitive assignment awaits their species designation. Determining this, however, may prove problematic because they reproduced the previously identified hybrid nature of many simian enterovirus genomes (9, 10); variants such as A13 were identified as showing closer sequence relatedness to one species in the capsid-encoding region (*EV-A*) and to another in the NS region (*EV-B*). The subsequent characterization of further simian viruses, the identification of subgroups with species A (A1 and A2 [26]) and B (human viruses and SA5) and the new complete genome sequence data from mandrill derived viruses provided the opportunity for a reevaluation of the occurrence of recombination in the genomes of simian (and human) viruses, from which various species classification possibilities might be proposed.

**Genome configurations.** Despite the seeming complexity of sequence relationships between simian and human viruses in different genome regions (Figure 4 to 6 and Figure 6 in reference 9), structural and nonstructural gene regions of all OWM monkey-derived viruses ultimately show only three genome configurations.

**(i) Group 1.** Group 1 comprises SV19, SV43, SV46, and EV92 which form part of the A2 subgroup in species A, along with the human viruses EV76 and EV89 to - 91 (that may indeed be zoonotically acquired) (26).

**(ii) Group 2.** Group 2 comprises A13 (in species A2 in P1), *EV-J*, and the mandrill viruses. All three possess P1 sequences classified as or closely related to *EV-A* with



pairwise distances below (A13 and mandrill viruses) or around the species assignment threshold (*EV-J*) of 40% for this region (Table 2; Figure 4B). However, all of these viruses possess species B-like NS gene regions, differing from each other and from *EV-B* by 6% to 21% in the 2C plus 3CD region and a well-defined breakpoint at the P1/P2 boundary (Figure 4B; see Figure S1 in the supplemental material). This commonality is further manifested by similar distributions of pairwise distances to other EV species (Figure 4B and Figure S2).

**(iii) Group 3.** Group 3 is represented by SA5 that is most closely related to human *EV-B* sequences throughout its genome. Several further examples of this group potentially exist among samples obtained in the current study from mandrills. Group 3 viruses likely represent the source of the species B-like NS gene regions of group B viruses, through recombination, accounting for the remarkably similar 3CD sequences of SA5 and mandrill viruses (6% divergence).

As previously discussed (9, 33, 37), there is further recombination between coding regions and 5'UTRs and 3'UTRs within human and simian enteroviruses. Sequence relationships in the 5'UTR and 3'UTR are distinct, although both regions show different propensities for species mergers and splits. For example, human species A1 and B variants are phylogenetically interspersed in the 5'UTR, as are species C and D along with some A2 viruses, while *EV-J*, simian *EV-B* (SA5) and mandrill viruses group closely together in the 3'UTR. A common feature, however, is the universally distinct grouping of simian- and human-derived viruses from each other in both regions. This observation is consistent with the existence of host range determinants in these genome regions and their potentially limiting effect on the occurrence of zoonotic infections. Further genetic characterization of simian viruses and *in vitro* studies are required to formally examine this hypothesis.

**Enterovirus classification.** The final classification of mandrill and other OWM viruses obtained in the current study may necessitate an update to the current species assignment criteria (1). By definition, viruses such as those obtained in the current study, A13 and *EV-J* which show evidence for interspecies chimerism between capsid and NS gene regions cannot simultaneously fulfill criteria 2 (< 40% divergence in P1)

and 3 (< 30% divergence in 2C plus 3CD) for any one species. This phenomenon similarly limits the use of divergence thresholds for complete coding region sequences (criterion 1). As the capsid likely possesses elements that primarily determine EV host and cellular tropisms and other biological properties, it may perhaps be appropriate to regard P1 divergence as the primary criterion for species assignment, although this will require further discussion with the International Committee for the Taxonomy Study Group.

Findings in the current study additionally highlight the need to reexamine the species assignment threshold for P1, currently set at 40%. Pairwise distances between *EV-A* (particularly A2) types, A13, mandrill viruses, and *EV-J* are mostly below this threshold (Figure 4B), and simian viruses in these groups all possess a common (*EV-B*-like) NS gene region (and mostly group together in the 3'UTR). A slight elevation of the 40% threshold would allow their assignment as species A and would substantially simplify the current classification of simian EVs (i.e., all members of groups 1 and 2 described above would be classifiable as *EV-A*). Although this would create a species containing both nonrecombinant and recombinant viruses, this is not different from *EV-C*, which also contains variants with evidence for chimerism in NS gene regions and in the 5'UTR (31, 32).

The alternative would be to lower the P1 threshold 40% to 32% so that *EV-J* and the mandrill viruses could be classified as separate species. The immediate problem with this is that this reduction would have the undesirable effect of splitting species C and A. As described above, the other problem is that structural gene sequences of *EV-A*, *EV-J*, and mandrill viruses fail to form clearly resolvable monophyletic clusters when included in the same data set (Figure 3A and B), and bootstrap support for these groupings requires the whole P1 region (see Figure S3 in the supplemental material). The diversity of simian viruses is likely considerably undersampled, and it is likely that further viruses showing loose sequence affiliations to these variants will be characterized in the future and create further complications with *EV-J* and mandrill EV species assignments.

Overall, this study provides substantial new information on the genetic diversity and prevalence of naturally occurring enteroviruses in wild primate populations. It is increasingly clear that relationships between variants infecting humans and nonhuman primates are complex, notwithstanding the growing evidence for zoonotic infections in both directions (12, 38) and continuing uncertainty about the timescales for the original divergence of types and species and appearance of interspecies chimeras. Understanding which genome regions ultimately determine host range and disease outcomes are essential in assessment of the risk of simian enteroviruses as potential zoonotic and pathogenic infections in humans.

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## Chapter conclusion

The study described in this chapter identified both known and novel EVs naturally circulating in wild monkeys with remarkable genetic diversity. The chimeric nature of novel EV genomes presents additional difficulties for their classification into types and species which will require re-examination of the current species demarcation criteria of the *Enterovirus* genus. None of the identified EVs overlapped in host range with those reported from humans despite the close genetic relationship of primates as well as increasing evidence of other EVs capable of infecting both NHPs and humans. For EVs detected in both humans and NHPs in previous studies (Harvala *et al.*, 2011b, Oberste *et al.*, 2013a, Oberste *et al.*, 2013b, Harvala *et al.*, 2014, Sadeuh-Mba *et al.*, 2014), further analyses (e.g. time scaled trees in BEAST) can be performed to understand the direction of EV transmission between human and non human hosts.

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## Chapter 6. Genetic characterization of picornaviruses in rodents and civets

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### 6.1. Introduction

#### 6.1.1. Rodents and civets as hosts of zoonotic pathogens

Rodents are members of *Rodentia*, the largest order of mammals. With over 2,200 living species including rats, mice, voles, prairie dogs, squirrels, porcupines, beavers, chipmunks, and guinea pigs, *Rodentia* comprises about 43% of all mammal species (Huchon *et al.*, 2002). Many rodents live in close proximity to humans, thereby serving as a potential source of zoonotic infection in humans (Nguyen *et al.*, 2015). Rodents have been known to host pathogens that cause more than 60 human diseases (Meerburg *et al.*, 2009) and viruses with unknown zoonotic potential (e.g. HuVs, kobuviruses, rosaviruses). Pathogens from rodents can be transmitted to humans either directly or indirectly through their saliva, urine, faeces or via their arthropod ectoparasites such as ticks, mites, and fleas (Meerburg *et al.*, 2009). A summary of the main zoonotic diseases caused by viral agents transmitted from rodents to humans are listed in Table 6.1. A few cases of human infection with picornaviruses such as EMCV-1 (see section 6.1.2) and FMDV, which can also infect rodents, have been confirmed by virus isolation and detection of a specific immune response (Brown, 2001).

**Table 6.1.** Viral pathogens that may be transmitted by rodents to humans, adopted from (Meerburg *et al.*, 2009)

Disease	Agent
Hantavirus Pulmonary Syndrome	<i>Bunyaviridae</i>
Hemorrhagic Fever with renal syndrome (+ other hemorrhagic fevers)	<i>Bunyaviridae</i>
Nephropathia epidemica	<i>Bunyaviridae</i>
Crimean-Congo hemorrhagic fever	<i>Bunyaviridae</i>
Borna disease	<i>Bornaviridae</i>
Omsk hemorrhagic fever	<i>Flaviviridae</i>
Kyasanur Forest Disease	<i>Flaviviridae</i>
Apoi Virus Disease	<i>Flaviviridae</i>
Tick-borne encephalitis	<i>Flaviviridae</i>
Powassan encephalitis	<i>Flaviviridae</i>



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Lymphocytic Choriomeningitis virus	<i>Arenaviridae</i>
Lassa Fever	<i>Arenaviridae</i>
South American arenaviruses	<i>Arenaviridae</i>
North American arenaviruses	<i>Arenaviridae</i>
Colorado Tick Fever	<i>Reoviridae</i>
Venezuelan equine encephalitis	<i>Togaviridae</i>
Western equine encephalitis	<i>Togaviridae</i>
Hepatitis E	<i>Caliciviridae</i>
Cowpox	<i>Poxviridae</i>
Contagious viral animal diseases (Classical Swine Fever, Foot and Mouth Disease)	<i>Picornaviridae</i> (FMDV); <i>Flaviviridae</i>

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Although no picornaviruses have been reported from civets, these animals were identified as a transmitting host of pathogens including those that have an important impact on human health such as H5N1 (Peiris *et al.*, 2007) and SARS coronaviruses (Wang & Crameri, 2014).

### 6.1.2. Kobuvirus, cardiovirus and HuV infection in rodents

Viruses within genera *Cardiovirus*, *Kobuvirus* and *Hunnivirus* that can infect rodents include members of *Cardiovirus A* (EMCV-1 and -2), *Cardiovirus B* (TMEV, TRV), *Cardiovirus C* (Boone cardiovirus 1 and 2), *Aichivirus A* (murine kobuvirus 1), and *Hunnivirus A*. These viruses are generally host-specific and distinct from members within these genera exclusively detected in humans (VHEV and SAFV in *Cardiovirus B*, AiV-1 in *Aichivirus A*). A prominent exception is EMCV-1, which has been isolated from over 30 host species including humans (Knowles *et al.*, 2010). However, whether the infrequent detection of EMCV-1 (as well as VHEV) in humans truly represents a genuine infection has been questioned, and it has been proposed that its presence in cell culture isolates arose through contamination of the cell lines or reagents used for isolation of the viruses (Lipton, 2008; Oberste *et al.*, 2009).

The pathogenicity of members of these genera is incompletely characterised, although kobuviruses are known to cause gastroenteritis in humans, cattle and swine (Reuter *et al.*, 2011). The pathogenicity of HuVs remains to be determined. Meanwhile, infection with the cardioviruses, TMEV and EMCV results in a rapidly fatal encephalitis or persistent infection in the central nervous system of mice (Lehrich *et al.*, 1976; Lipton, 1975).

### 6.1.3. Rodent and civet consumption in Vietnam

Rodent meat is a popular source of protein for consumption by humans in Vietnam, particularly in the Mekong Delta, where rats are commonly sold live in wet markets (Nguyen *et al.*, 2015). The total annual consumption of rat meat in Vietnam is 3,300 – 3,600 tonnes (Khiem *et al.*, 2003). As rodents are reservoirs or carriers of a significant number of zoonotic pathogens (Meerburg *et al.*, 2009) and viruses with unknown zoonotic potential, there are potential and demonstrated health risks associated with exposure to these animals. A previous study (Khiem *et al.*, 2003), however, showed none of the surveyed rat catchers or processors were aware of infection risks from contact with live rats. Consequently, no precautions were taken for the handling of rodents (Figure 6.1).



**Figure 6.1.** Direct contact during handling of rats without protection measures puts handlers at risks of acquiring zoonotic infections.

Moreover, Vietnam now has a growing demand for wild animal products as a result of a rising population and increasing urban prosperity (Drury, 2011). This leads to commercial wildlife farming (such as civets, porcupines, bamboo rats, wild boars, snakes) as a possible solution to meeting that growing demand (Cooper, 1995). The Asian palm civets (*Paradoxurus hermaphroditus*) are additionally farmed for the production of civet coffee (Deutsch & Murakhver 2012) during which the cherry coffee beans pass through the civet's digestive tract before being defecated with other faecal matter and collected. Meanwhile, human exposure to livestock is known as an

important risk factor for cross-species transmission of zoonotic pathogens (Mackenzie, 2005; Pham *et al.*, 2006).

In assessing the potential zoonotic risks of picornaviruses to humans, 437 faecal samples from rodents and civets in Vietnam were screened in Chapter 3. Screening showed detection of kobuviruses in 103/407 rodents, 5/30 civets; cardioviruses and HuVs in 27/407 and 54/166 screened rodents, respectively. This chapter provides further information on the prevalence of infections in the screened animal species, the genetic diversity of these viruses and their relationships to those documented from humans. The hypothesis of this chapter is that these animals may carry picornaviruses that can also infect humans.

## **6.2. Materials and methods**

### **6.2.1. Materials**

RNA extracted from rodent and civet positive samples (Chapter 3) was used for characterisation of viruses in this chapter.

### **6.2.2. Identification of rodent species**

Rodent species were identified on the basis of biological and morphological characteristics and confirmed by sequencing of a housekeeping gene (COI) (Chapter 2).

### **6.2.3. VP4/VP2, VP1 amplification**

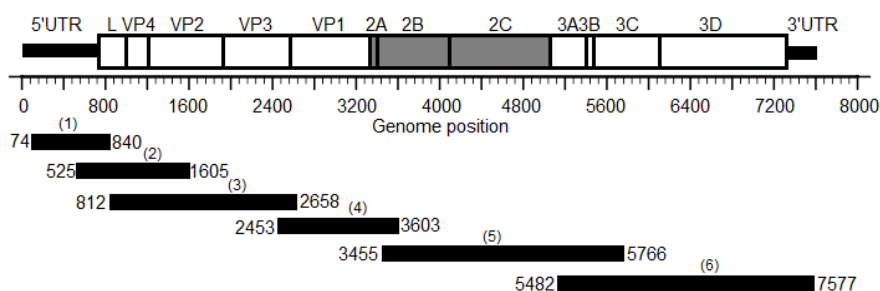
Characterization of viruses in positive samples was performed by amplification and sequencing of VP4/VP2 and VP1 regions. For these regions, the RT step was combined with the first round PCR using Superscript III one-step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, UK) in a total volume of 20 µl containing 6 µl of extracted RNA. Cycling conditions were 45°C for 30 min, 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, 68°C for 1 minute/kb. Then 1 µl of the first-round reaction was used for the nested PCR with second-round primers and GoTaq DNA polymerase (Promega, UK). PCR amplification included

95°C for 5 min followed by 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and elongation (72°C, 90 s) in a thermal cycler.

VP4 and VP1 PCR products were sequenced on both strands using the BigDye Terminator v3.1 (Applied Biosystems) and primers for second round PCR. All primers used for screening and genetic characterization of viruses in this study are listed in Table 2.6 (Chapter 2).

#### 6.2.4. Complete genome sequencing of novel HuVs

Additional primers were designed to amplify regions of up to 2.2 kb in length with overlaps (Table 2.5; Chapter 2; Figure 6.2). The RT was combined with the first round PCR as described for VP4/VP2, VP1 above. SequalPrep long PCR kit (Life Technology, UK) was used for second round PCR according to the manufacturer's instruction instead of GoTaq DNA polymerase to amplify fragments longer than 2 kb.



**Figure 6.2.** Schematic diagram of the HuV genome and amplified fragments (in parentheses) with numbers indicating the positions of each fragment for complete genome of novel types.

#### 6.2.5. Sequence analysis

Sequences were first imported into DnaBaser ([www.DnaBaser.com](http://www.DnaBaser.com)) for assembly before import into SSE (Simmonds, 2012) for alignment and calculation of sequence uncorrected distance against reference sequences. Maximum Likelihood trees were constructed using the MEGA 5.2 software package (Tamura *et al.*, 2011) with 1000 bootstrap re-samples and the best fit models (general time-reversible, gamma distribution for cardiovirus, kobuvirus and HuV VP1, Tamura–Nei, gamma distribution for cardiovirus VP4/VP2). Amino acid p distance scan implemented in SSE was applied to show variability across a genome. Distance values were calculated using a window size of 300 bases, incrementing by 30 bases between windows.

Sequences obtained from this chapter have been deposited in GenBank under accession numbers KT944114 – KT944214.

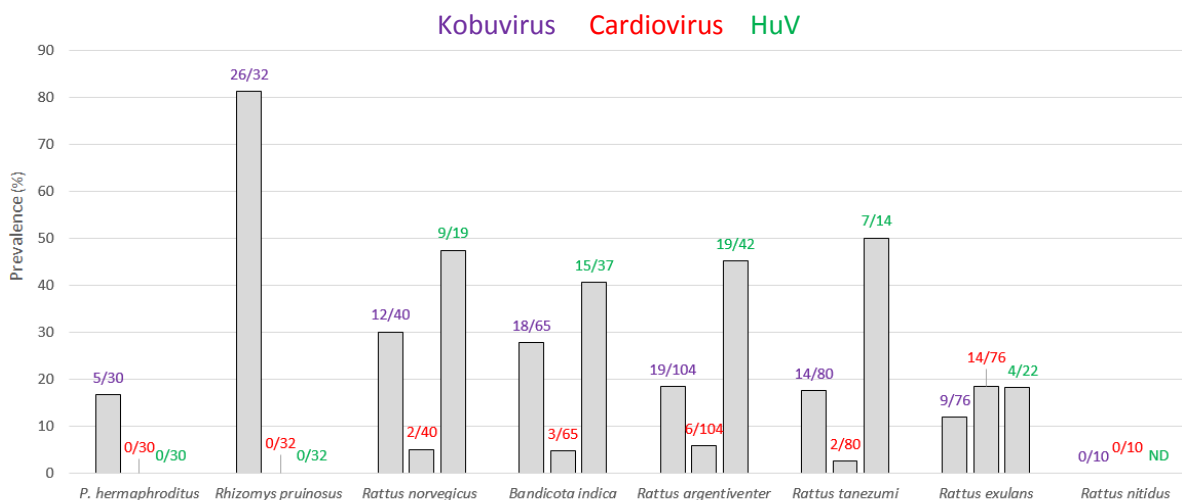
### 6.3. Results

#### 6.3.1. Species identification

Species identification showed that the 407 rodents belonged to seven species. Among these, *Rattus argentiventer* was most frequently identified (n = 104; 25.6%) while the least frequently sampled species, *Rattus nitidus*, accounted for 2.5% (10/407) of samples. All 32 bamboo rats were members of the species *Rhizomys pruinosus* (Figure 6.3).

#### 6.3.2. Prevalence of picornavirus RNA

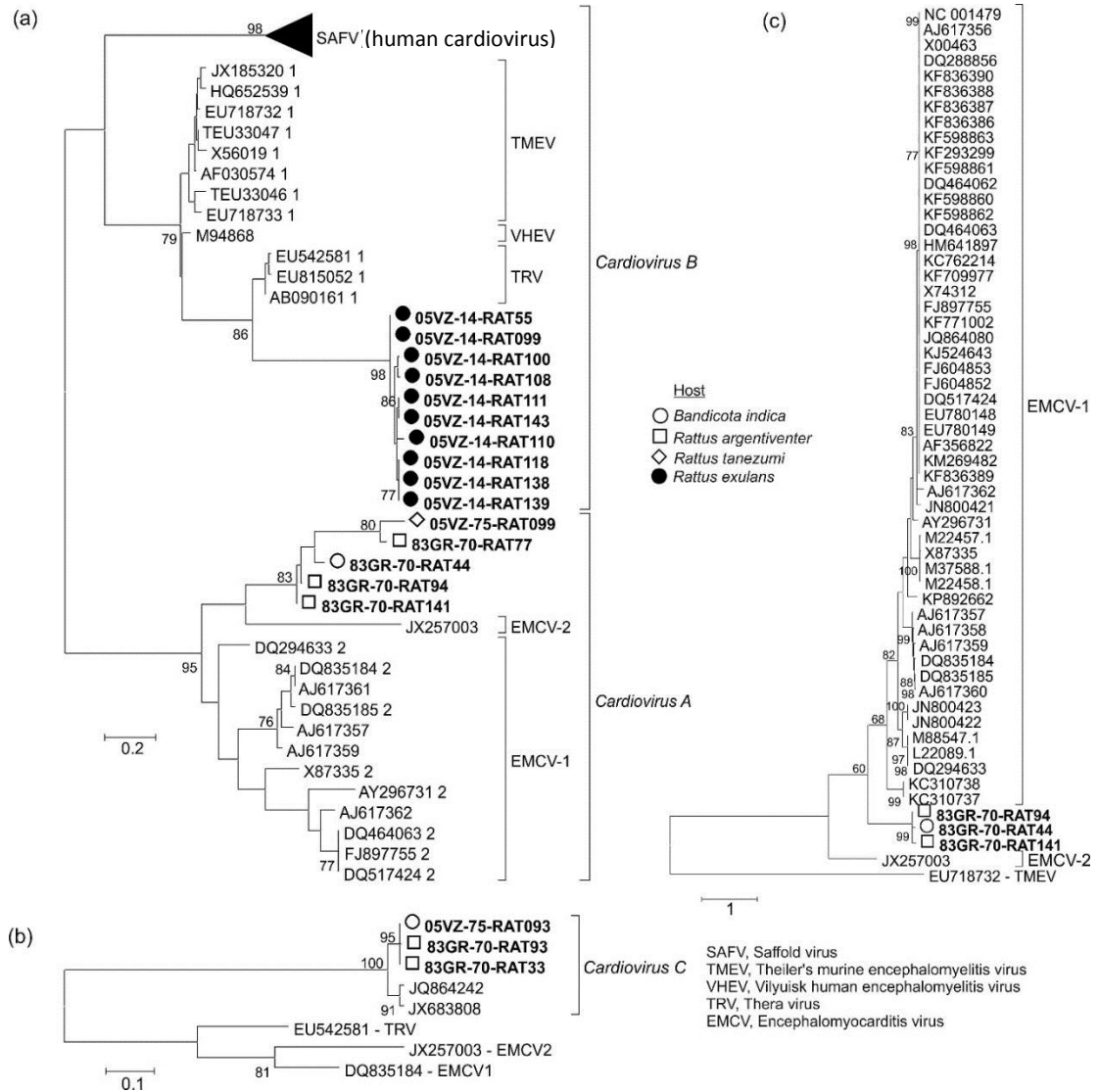
Kobuvirus RNA was the most commonly detected viral RNA, detectable in 16.7% (5/30) of civets and 25.3% (103/407) of rodents. Cardiovirus and HuV RNA was only found in rodents with detection frequencies of 6.6% (27/407) and 32.5% (54/166), respectively. Six of seven rodent species were positive for the targeted picornaviruses with infection frequencies varying according to rodent species and viruses but with consistent absence of the screened viruses in *Rattus nitidus*. Kobuvirus RNA was most common in *Rhizomys pruinosus* (81.3%; 26/32) while cardiovirus and HuV RNA was more commonly found in *Rattus exulans* (18.4%; 14/76) and *Rattus tanezumi* (50%; 7/14), respectively, than in other rodent species (Figure 6.3).



**Figure 6.3.** Prevalence of the screened picornaviruses by hosts. Columns were labelled with number of positive samples / number of samples tested for kobuvirus (in purple), cardiovirus (in red) and HuV (in green). ND, not done.

### 6.3.3. Genetic characterization of picornaviruses in rodents and civets

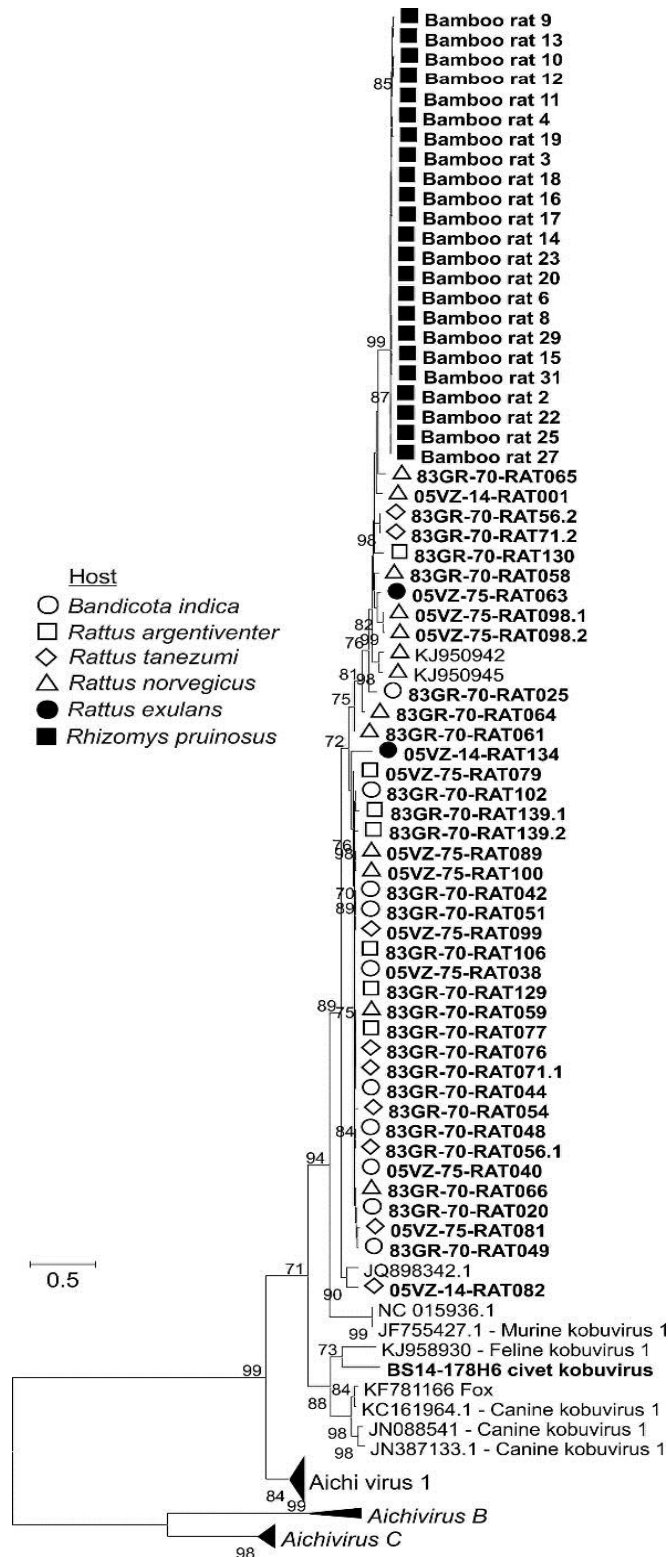
To genetically characterize picornaviruses in positive samples, sequences were amplified from the VP4/VP2 region of cardioviruses and VP1 of all viruses. VP4/VP2 sequences were obtained from 18 of the 27 rodents positive for cardiovirus. Of these, three clustered with sequences of Boone cardioviruses, five sequences grouped with EMCV and ten were most similar to TRV sequences. All of these sequences were separate from human cardioviruses (SAFV) (Figure 6.4a). Despite attempts to amplify the VP1 region of cardioviruses in positive samples, only three variants, assigned as *Cardiovirus A* based on VP4/2 region sequences could be successfully obtained in this region. VP1 sequences confirmed their species assignment (Figure 6.4b) although they all showed substantial nucleotide sequence divergence from EMCV-1 reference sequences (p distances of 32.4 – 38.6%) and grouped more closely with EMCV-1 than EMCV-2.



**Figure 6.4.** Phylogenetic relationships between cardioviruses detected in Vietnamese rats (highlighted in bold) and reference sequences in the regions (a) and (b) VP4 (nt 1295 – 1583) and (c) VP1 (nt 3005 – 3832).

Sixty three VP1 sequences could be amplified from 1 of 5 civets and 58 of 109 rodents positive for kobuviruses, including four co-infections in rats (Figure 6.5). Bamboo rat derived kobuvirus sequences were highly similar to each other (nucleotide sequence distances in the range of 0 – 2.9%). These sequences formed a group that clustered closely with nucleotide sequence distances of 11.6 – 23.6% to other Vietnamese rat kobuviruses. Interestingly, kobuvirus VP1 sequences recovered from five rat species grouped together and showed nucleotide sequence distances of 0 – 21.8%. One sequence derived from a civet was most similar to feline kobuvirus. All of the sequences in this study additionally formed a large group with reference sequences

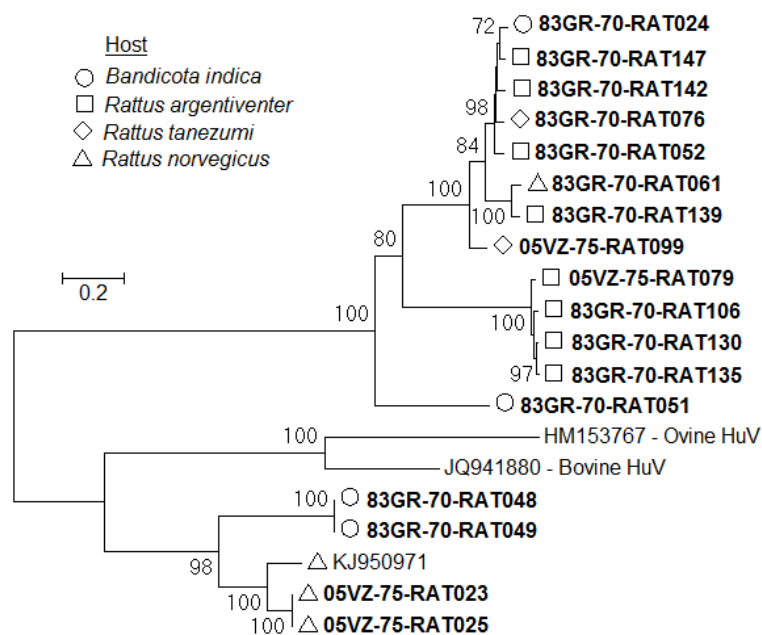
which were previously reported from animals and recently classified as members of species *Aichivirus A*.



**Figure 6.5.** Phylogenetic relationships between kobuvirus VP1 (nt 3005 – 3838) sequences detected in Vietnamese rats and a civet (highlighted in bold) and reference sequences.



In contrast, seventeen HuV VP1 sequences obtained from rats were more divergent, even those from the same rat species (Figure 6.6). Four of these sequences grouped with a sequence (KJ950971) from New York rats (nucleotide sequence distances of 14.5 – 28.3%) while the others formed a group separate from all known sequences available from GenBank. Sequence distances between the latter group and reference sequences were 45.5 – 49.3%, indicating the existence of novel, highly divergent HuVs in these rodent populations.



**Figure 6.6.** Phylogenetic relationships between HuV VP1 (nt 2597 – 3298) sequences detected in Vietnamese rats (highlighted in bold) and reference sequences.

#### 6.3.4. Analysis of HuV coding region complete sequences

Almost complete genomes of three novel HuVs (05VZ-75-RAT099, 83GR-70-RAT106, 83GR-70-RAT130) were sequenced and further characterized. An amino acid distance scan (Figure 6.7) demonstrated similar divergence patterns across the coding region between the obtained genomes and each reference sequence. In the P1 region, the sequence distance of Vietnamese HuVs compared to New York HuV (35.4%) was almost as high as to ovine (37.5%) and bovine HuVs (36%). From the border of P1/P2, however, Vietnamese HuVs were substantially less divergent from New York rat HuV than the ovine and bovine HuVs. The combined 2C + 3CD region of Vietnamese HuVs was 3.9%, 18% and 18.6% different from the reference sequences from rats, oxen and sheep, respectively. Remarkably, sequence distance

scan also showed that the 2B region was substantially more variable than the other non-structural proteins (Figure 6.7). This divergence pattern is a distinct feature of HuV genomes in comparison with other picornaviruses whose non-structural proteins are relatively equally conserved.

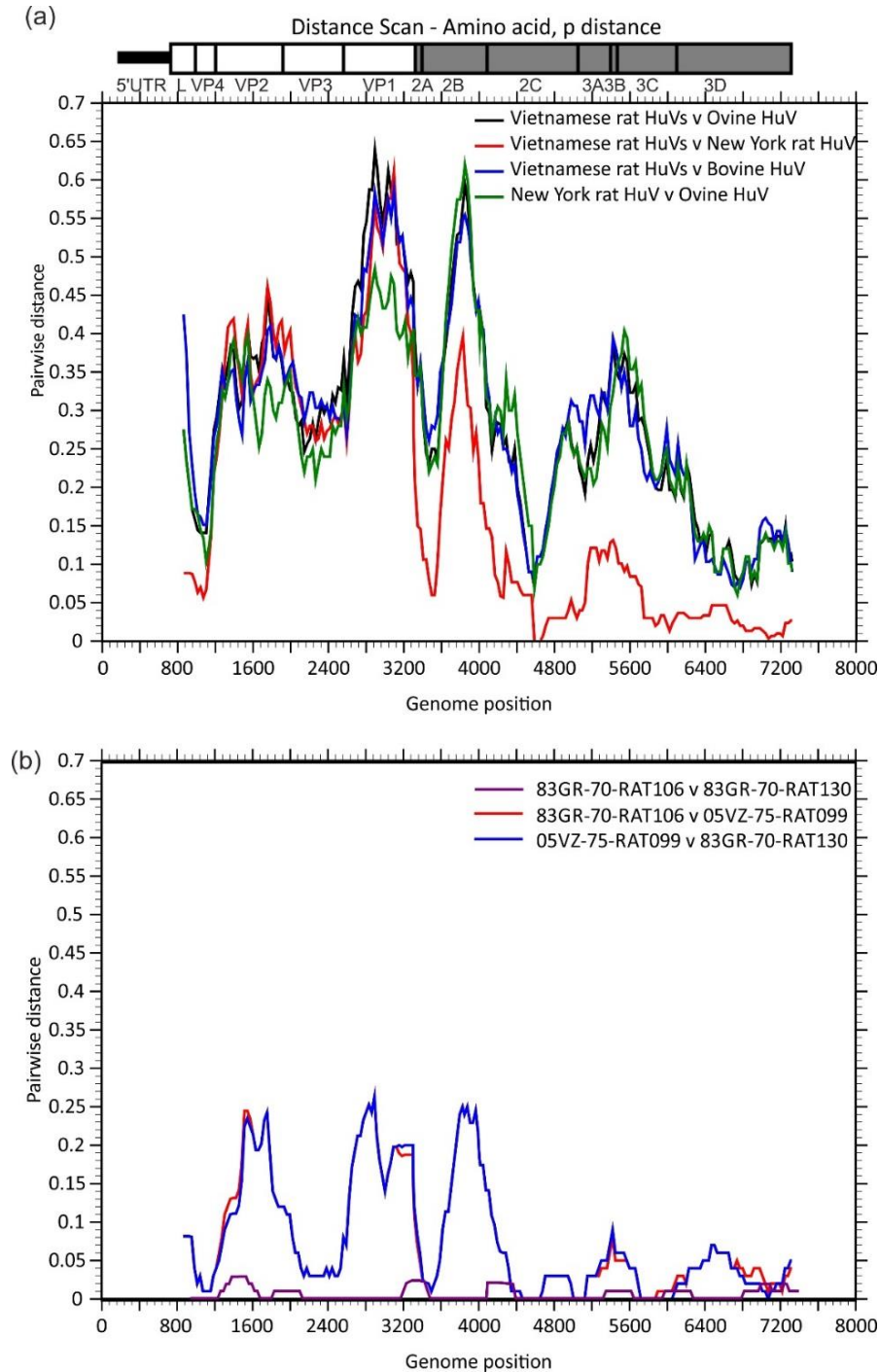


Figure 6.7. Amino acid distance scan across the coding region of (a) Vietnamese HuVs against reference sequences and (b) between Vietnamese HuVs.

## 6.4. Discussion

### 6.4.1. Prevalence and host range of the screened picornaviruses

This is the first large scale study investigating kobuviruses, cardioviruses and HuVs in rodents and civets. The absence of cardioviruses and HuVs in bamboo rats and civets was consistent with the fact that these viruses have not been reported from these animals. However, studies in this chapter have documented, for the first time, the relatively frequent detection of cardioviruses in civets, kobuviruses in bamboo rats and kobuviruses, HuVs in five different rat species. These findings provided new information on the existence of these viruses in a much wider range of hosts than previously documented. Before this work, only one study (Firth *et al.*, 2014) reported the prevalence of kobuviruses and HuVs in rat (*Rattus norvegicus*) faecal samples of 50% and 19%, respectively. These figures contrast with the corresponding detection frequencies of 30% and 47.4% in the current study. Kobuvirus infection in bamboo rats was more common with a frequency of 81.3% (Figure 6.3). Firth *et al.* additionally reported prevalence of 28% (37/133) and 14% (19/133) of Boone cardiovirus and TRV respectively while the overall detection frequency of cardioviruses in *Rattus norvegicus* in this study was 5% (2/40). Although rodents are thought to be the natural host of EMCV-1 (Tesh & Wallace, 1978), little is known about its prevalence in wild rodent populations as only seroprevalence studies, not molecular surveillance, have been conducted, mostly in response to encephalomyocarditis outbreaks in pigs and other non-rodent mammals (Philipps *et al.*, 2012).

### 6.4.2. Classification and zoonotic potential of the detected picornaviruses

Viruses in positive samples were further characterized by amplification and sequencing of capsid coding regions. VP1 sequences were successfully amplified from only 51.8% (59/114) of samples positive for kobuviruses (Figure 6.5), and 31.5% (17/54) of HuV positive rat samples (Figure 6.6). This may be because the primers were designed using a relatively limited number of reference sequences from similar viruses available in GenBank and which did not match all kobuvirus and HuV sequences present in the samples. These primers should be re-examined and validated before use in future studies when more sequence data are available.

Characterization by sequencing of the VP1 region indicated low genetic diversity of kobuviruses infecting Vietnamese rodents. Highly similar kobuvirus VP1 sequences recovered from five rat species and bamboo rats (Figure 6.5) suggested that these viruses have low host species specificity, and they could cross the species barrier, to transmit between different rodent species. This is a characteristic that may lead to its establishment and emergence in new hosts. Understanding the receptor usage for cell entry of kobuviruses in rodents and other host species would potentially help predict the host range of these viruses. Despite the ability to infect different rodent species and VP1 sequences clustering in species *Aichivirus A*, the identified rodent kobuviruses were distinct from human kobuvirus (AiV-1). Whether these variants can be classified as different serotypes is currently uncertain. As previously discussed (Nguyen *et al.*, 2015), VP1 sequence distance thresholds for assignment of (sero)types may be different between picornavirus species even in the same genera. This is demonstrated by (sero)type assignment thresholds of  $> 25\%$  in the VP1 nucleotide sequences of *Enterovirus A – D* and but only  $\geq 12 – 13\%$  of *Rhinovirus A – C* of genus *Enterovirus*. Sequence distances of kobuvirus VP1 lie between these two thresholds and their antigenic / neutralisation properties that might define serotype specificity cannot be readily predicted.

In contrast to kobuviruses, VP1 sequences of the novel HuVs were considerably divergent (45.5 – 49.3%) from previously known sequences (Figure 6.6). The amino acid distances of the three novel HuV variants against the ovine and bovine HuV sequences (GenBank accession numbers HM153767 and JQ941880) of 36 – 37.5% in P1 and 18 – 18.6% in the combined 2C + 3CD regions were comparable to those corresponding figures of 31.4 – 32.1% and 18.6 – 19.3% of the New York rat HuV (KJ950971). The novel HuVs may, therefore, represent new genotypes/(sero)types of *Hunnivirus A* or potentially new species. More information on the patterns of genetic variability and host associations of this genus is required, however, to establish degrees of divergence appropriate for these taxonomic divisions.

Cardioviruses in the positive samples could be identified as variants of Boone cardioviruses (including *Bandicota indica* and *Rattus argentiventer* derived sequences), TRV like viruses (from *R. exulans*) and EMCV (from *B. indica*, *R.*

*argentiventer* and *R. tanezumi*) on the basis of the VP4/VP2 region (Figure 6.4). This is an evidence for a wider host range of Boone cardioviruses and TRVs which had previously been documented from *R. norvegicus* (Drake *et al.*, 2008; Firth *et al.*, 2014; Gohndrone & Riley, unpublished).

In spite of being isolated from various hosts including mouse, hamster, chimpanzee and rhesus monkey, EMCV strains are serologically identical (Dick, 1949; Warren *et al.*, 1949) and were therefore assumed to belong to a single serotype (EMCV-1) (Knowles *et al.*, 2012). The second EMCV serotype (EMCV-2) was discovered in a wood mouse and described seven decades after the first description of EMCV (Philipps *et al.*, 2012). More recently, Yeo *et al.* reported sequences (KC310737, KC310738) from two isolates that reacted to polyclonal antiserum raised to EMCV and showed closer VP1 sequence distances to EMCV-1 than EMCV-2 (Yeo *et al.*, 2013), indicating they could be variants of EMCV-1. These sequences represent the most divergent sequences of EMCV-1 described to date with maximum nucleotide and amino acid distances of 29.1% and 15%, respectively, to all other known EMCV-1 sequences in the VP1 region. In the current study, only three cardiovirus VP1 sequences could be amplified from 18 samples positive by VP4/VP2 PCR. The high sequence divergence in the VP1 region may have resulted in amplification failure in most of the positive samples. The obtained VP1 (Figure 4b) sequences clustered most closely to EMCV-1 with sequence distances of 32.4 – 38.6% and 28 – 32.7% to EMCV-1 sequences at the nucleotide and amino acid levels, respectively. As these distances are remarkably higher than those of within EMCV-1 sequences, a neutralization test would be needed to confirm the serotype assignment of Vietnamese rat cardioviruses as EMCV-1 or a new serotype.

In conclusion, the study in this chapter demonstrated the existence of kobuviruses, HuVs and cardioviruses in different host species of Vietnamese animals from most of which these viruses had never been reported. Although similar sequences were recovered from different rodent species, the identified kobuviruses as well as cardioviruses were genetically different from viruses reported from humans to date and may therefore have no zoonotic potential. This study additionally highlighted the incomplete characterization of the genetic diversity of genus *Hunnivirus* by detection

of sequences highly divergent from all previously known sequences. The study reported in the next chapter using human samples from high risk cohort will elucidate if any zoonotic events relating to the targeted picornaviruses occurred in Vietnam over the course of this thesis.

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## Chapter 7. Genetic characterization of picornaviruses in human samples

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### 7.1. Introduction

#### 7.1.1. Detection of EVs, kobuviruses and cardioviruses in humans

Among twelve species of genus *Enterovirus*, members of seven species can infect humans (*EV-A* to *-D*, *Rhinovirus A* to *C*). Except for rhinoviruses currently unique to humans, *EV-A* to *-D* additionally include viruses that can also infect NHPs and pigs or have only been reported from NHPs (more details presented in Chapter 1). Similarly, kobuviruses detected from humans belong to species *Aichivirus A* with recent recognition of animal kobuviruses (canine kobuvirus 1, feline kobuvirus 1 and murine kobuvirus 1) as members of this species (<http://www.picornaviridae.com/kobuvirus/-kobuvirus.htm>). Meanwhile, VHEV and EMCV-1 have been infrequently isolated from human samples (Goldfarb & Gajdusek, 1992; Kirkland *et al.*, 1989; Oberste *et al.*, 2009; Verlinde & Van Tangeren, 1953), and SAFV is a novel cardiovirus reported from patients without disease manifestations attributable to the virus (Himeda & Ohara, 2012). Comparison of viruses identified from animals in my studies in Chapters 4 – 6 with those documented from humans to date revealed no overlaps. This chapter characterises picornaviruses in human positive samples from high risk populations due to their frequent occupational exposure to animals to determine whether any zoonotic events occurred in the studied populations.

#### 7.1.2. People at high risk of zoonotic infections

All humans are at some risk of zoonotic infections, but some populations are at higher risk (Pimentel & Taylor, 2015). Lack of understanding of how diseases spread, frequent hand-to-mouth contact, and improper hand washing put children at increased risk of acquiring zoonoses. Immunocompromised individuals, such as HIV/AIDS patients, organ-transplant recipients or pregnant women, are also more likely to be infected with zoonotic pathogens. In addition, as mentioned in Chapter 1, the continuum of contacts between humans and animals has created the human–animal

interface, a key factor for cross-species transmission (Gortazar *et al.*, 2014). Most of zoonotic pathogens are transmitted through either direct or indirect contact (Woolhouse & Gowtage-Sequeria, 2005). Therefore, agricultural workers, veterinarians, market sales people, slaughterhouse workers, etc. who are in frequent and close contact with animals through such as bushmeat practice, the trading of animal foods, animal practices, wet markets, the domestication of animals have high risk of contracting zoonotic infections (Pike *et al.*, 2010). This results in an urgent need for co-ordinated surveillance of at-risk human populations and animals populations to which people are exposed.

In the VIZIONS project, participants are eligible for inclusion if they meet all the following criteria: i) they are involved in raising, slaughtering, or processing livestock or wildlife; ii) give informed consent for study participation; iii) are willing to have their animals sampled. Children < 1 year of age are not included.

### **7.1.3. High risk cohort in this study**

VIZIONS high risk human populations geographically matched to the screened animal samples were of interest. As most animal samples were taken in Dak Lak and Dong Thap, these two provinces were targeted for sampling of humans. The cohort members considered to be at high risk of acquiring a zoonotic infection as a consequence of occupational exposure to animals in this study included (1) farmers, (2) animal health workers, (3) abattoir workers, and (4) market sales workers. From this cohort, a total of 250 faecal samples including 100 from Dong Thap and 150 from Dak Lak provinces were collected and screened for picornaviruses in Chapter 3. The sampled people were aged from 21 – 64 years old. The screened samples (Table 7.1) were taken at enrolment (56 samples) or when cohort members developed illness (194 samples) such as fever, flu, acute pharyngitis, diarrhoea. Among these, there were samples positive for cardiovirus, kobuvirus and EV. This chapter characterises the picornaviruses in positive samples by amplification, sequencing and comparison of their informative genome regions (VP4/VP2, VP1) to reference sequences. Findings in this chapter will provide the answer for the hypothesis that the studied high risk populations may be infected with viruses detected in animals previous chapters.



**Table 7.1.** Number of human samples by occupation category

Occupation category	Number of sample
Farmer	104
Abattoir worker	73
Animal health worker	38
Market sales worker	35
Total	250

## **7.2. Materials and methods**

### **7.2.1. Materials**

RNA extracts of the positive samples were used for investigation of the virus characteristics.

### **7.2.2. Methods**

The full VP1 region of EV and kobuvirus isolates, and the VP4/VP2 region of cardioviruses in positive samples were amplified using one-step RT-nested PCR protocols. In case VP1 was not successfully amplified, EVs in positive samples were confirmed by sequencing of the 5'UTR. PCR products were checked using agarose gel electrophoresis before sequencing on both directions. Sequences were imported into SSE (Simmonds, 2012) for alignment and calculation of sequence distances from reference sequences. Maximum Likelihood trees were constructed using the MEGA 5.2 software package (Tamura, 2011) with 1000 bootstrap re-samples and best fitting models (Tamura and Nei model with gamma distribution for cardiovirus VP4/VP2; Hasegawa-Kishino-Yano model with gamma distribution for kobuvirus VP1; and General Time Reversible model with gamma distribution for EV VP1) as described in Chapter 2.

Sequences obtained in this chapter are presented in Appendix 2.

## **7.3. Results**

Screening of 250 samples from human high risk cohort by qPCR or nested PCR (Chapter 3) revealed 1, 1 and 8 samples positive for cardiovirus, kobuvirus and EV, respectively. In this chapter, characterizations of the positive viruses were performed

by amplification and sequencing of the VP4/VP2 or VP1 regions. One VP4/VP2 sequence was obtained from the sample positive for cardiovirus. The sequence clustered with human SAFVs with JN652233.1 (SAFV-2) as the most similar sequence (Figure 7.1a).

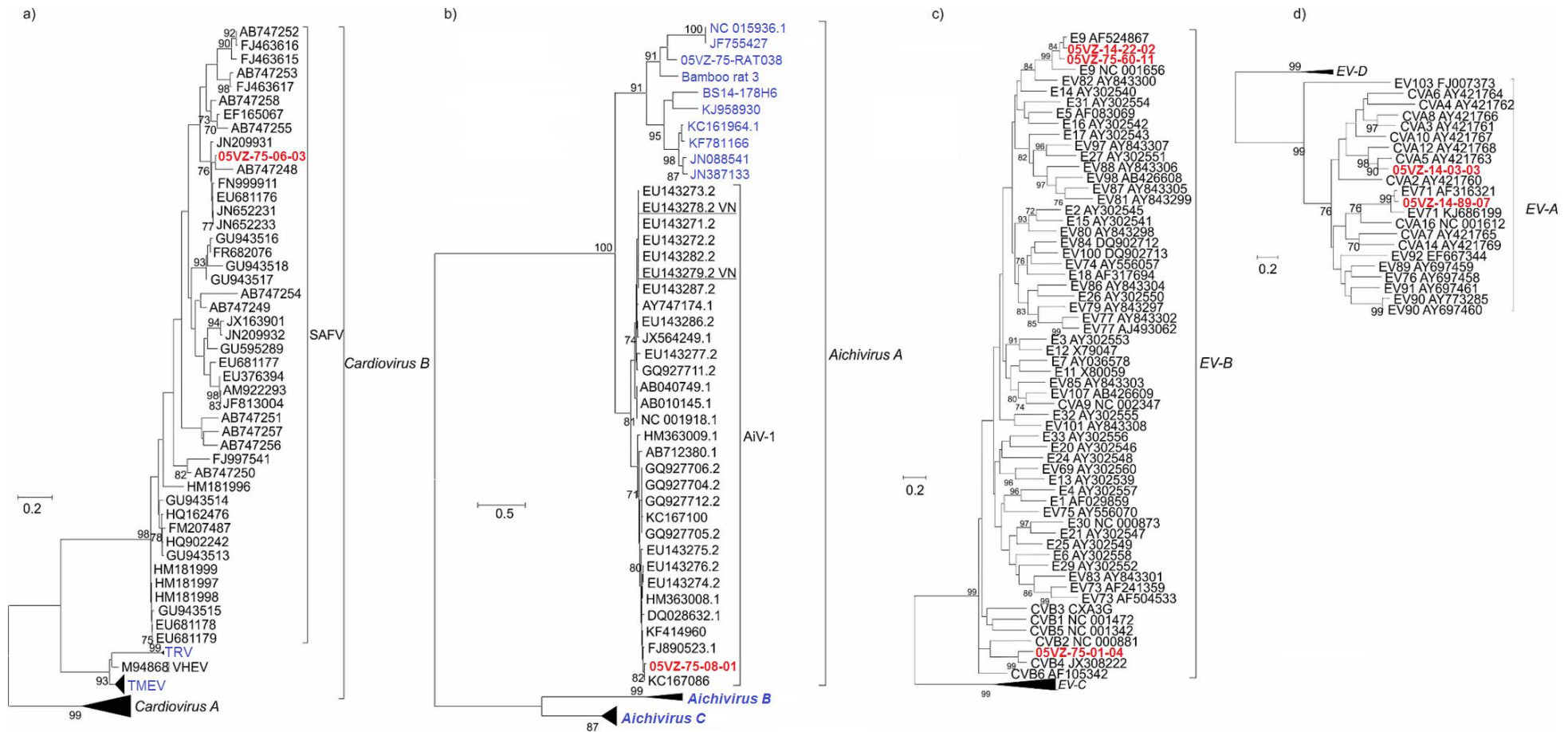
A kobuvirus VP1 sequence obtained from the single positive sample clustered with AiV-1 sequences in lineage II (Pham *et al.*, 2008) of species *Aichivirus A* (Figure 7.1b) with nucleotide sequence distances of 86 – 97%. The most similar sequence was KC167086.1 reported from a Korean patient (Han *et al.*, 2014).

VP1 sequences were obtained from 5 of the 8 EV positive samples (Figure 7.1c & d). Based on their grouping and nucleotide sequence distances (< 25%) to reference sequences in this genome region, EVs could be identified as variants of serotypes E9, CVB4, CVA5 and EV71. VP1 amplification failure in the 3 other samples may be due to low viral loads indicated by their high Ct values of 38 – 39. The BLAST results (performed on October 20, 2015; Table 7.2) of the 5'UTR sequences from these 3 samples showed 99 – 100% identity to EV71 sequences reported from Vietnam (KJ686199.1) and Thailand (KR045298.1). However, as recombination is frequently documented in this region (Haddad-Boubaker *et al.*, 2007; Simmonds & Welch, 2006), no EV types could be assigned for these viruses.

All of the identified picornaviruses have been documented from humans only. The infected patients were from all categories of occupation and most of them developed different clinical symptoms (Table 7.2).

**Table 7.2.** Characteristics of 10 cases from high risk cohort infected with picornaviruses

Patient ID	Age (years)	Occupation	Clinical symptoms	Collection date	Collection site	Virus	Highest identity (%)	Reference sequence	Country of the most similar sequence
05VZ-14-89-07	48	Poultry slaughterer	Flu-like	29/01/2015	Cu M'gar, Dak Lak	EV71	100%	HM358817.1	Malaysia
05VZ-14-67-02	25	Farmer	Flu-like	13/06/2015	Cu M'gar, Dak Lak	EV	99%	KR045298.1	Thailand
05VZ-14-20-02	31	Farmer	Flu-like	29/07/2015	Buon Ma Thuot, Dak Lak	EV	100%	KJ686199.1	Vietnam
05VZ-14-03-03	42	Farmer	Fever	10/03/2015	Buon Ma Thuot, Dak Lak	CVA5	95%	KP289363.1	China
05VZ-14-22-02	33	Farmer	Fever	24/09/2014	Buon Ma Thuot, Dak Lak	E9	98%	JN655888.1	China
05VZ-14-88-11	49	Poultry slaughterer	Diarrhoea, pharyngitis	12/08/2015	Buon Ma Thuot, Dak Lak	EV	100%	KJ686199.1	Vietnam
05VZ-75-01-04	24	Pig slaughterer	No (enrolment)	17/03/2013	Cao Lanh, Dong Thap	CVB4	95%	KF412987.1	India
05VZ-75-60-11	22	Pig slaughterer	No (enrolment)	02/06/2013	Cao Lanh, Dong Thap	E9	97%	AB746185.1	Japan
05VZ-75-08-01	37	Market sales worker	Gastroenteritis	19/04/2013	Cao Lanh, Dong Thap	AiV-1	97%	KC167086.1	South Korea
05VZ-75-06-03	25	Farmer	No (enrolment)	17/03/2013	Cao Lanh, Dong Thap	SAFV	96%	JN652233.1	USA



**Figure 7.1.** Phylogenetic analysis of a) cardiovirus VP4/VP2 region (nt 1295 – 1583), b) kobuvirus VP1 region (nt 2991 – 3821), c) and d) EV VP1 region (nt 2477 – 3376). Sequences obtained in the current study are highlighted in red. Reference sequences from animals and humans are highlighted in blue and black, respectively.

## 7.4. Discussion

### 7.4.1. Direct identification of viral RNA from clinical samples

In this thesis, RNA directly extracted from samples was screened in Chapter 3 using established real-time PCR or nested PCR protocols. Viruses in the positive human samples were characterized in this chapter by direct amplification and sequencing of informative genomic regions from the RNA extracts. This is in contrast with some previous studies which identified picornaviruses using virus isolation followed by amplification of the viruses. The isolation step was performed by inoculation of sample supernatants into animals or cell cultures (Kirkland *et al.*, 1989; Oberste *et al.*, 2009; Sarmanova & Chumachenko, 1960; Verlinde & Van Tangeren, 1953). The methods used in the previous studies presented several disadvantages. Firstly, virus isolation is laborious and time-consuming. Secondly, some viruses grow poorly or are even unable to grow in cell culture (Bochkov & Gern, 2012). Thirdly, detection of some viruses (e.g. VHEV, EMCV-1) in human samples (Kirkland *et al.*, 1989; Verlinde & Van Tangeren, 1953) turned out to be inconclusive and was suspicious of contamination from the used animals or cell lines (Lipton, 2008; Oberste *et al.*, 2009). These drawbacks could be overcome by direct detection and characterization of viruses from clinical samples using established real-time PCR and nested PCR protocols which have been proved as more sensitive, efficient and quick alternatives to the conventional methods (Kupila *et al.*, 2005).

The identification of virus types on the basis of the capsid gene regions using RT-nested PCR and sequencing additionally has advantages over neutralization tests for which antisera are in shortage of supply. Some studies used a one-step RT-PCR, consisting of only a first round PCR, for EV typing (Kiang *et al.*, 2009; She *et al.*, 2010). The authors suggested that contamination is less likely to occur in a “closed system”. However, VP1 amplification by the one-step RT-PCR was ineffective in about one third of tested isolates and significant non-specific products were also amplified in reactions with successful amplification of VP1 (She *et al.*, 2010). On the contrary, with a second round PCR, the one-step RT-nested PCR protocols used in this thesis greatly reduce non-specific amplification and are much more sensitive,

resulting in successful amplification of viruses with low titres. Furthermore, the one-way system with separate and dedicated lab spaces for each step (Chapter 2) efficiently prevents the occurrence of contamination.

#### **7.4.2. Genetic diversity and disease association of the identified picornaviruses**

With VHEV and EMCV-1 suspected of being contaminating viruses, SAFV may be the only genuine human cardiovirus. Despite high seroprevalence (75 – 90%) in both children and adults (Zoll *et al.*, 2009a), SAFV has been detected at low frequencies by molecular methods from patients with different illnesses such as fever of unknown origin (Jones *et al.*, 2007), respiratory tract infection (Abed & Boivin, 2008; Tsukagoshi *et al.*, 2011), gastroenteritis (Chiu *et al.*, 2010; Drexler *et al.*, 2008; Ren *et al.*, 2009), aseptic meningitis (Himeda *et al.*, 2011), sudden death syndrome (Nielsen *et al.*, 2012), nonpolio AFP and healthy children (Blinkova *et al.*, 2009). Taken together, these findings indicate SAFVs probably cause mainly asymptomatic infections or nonspecific symptoms and rarely result in severe disease (Nielsen *et al.*, 2012). SAFV was detected from a person with no clinical symptoms in the current study. This is the first report of SAFV in Vietnam.

AiV-1 has been extensively documented as a possible etiologic agent of human gastroenteritis in Japan (Yamashita *et al.*, 2000), Pakistan (Yamashita *et al.*, 1995), Japan, Bangladesh, Thailand and Vietnam (Pham *et al.*, 2007), France (Ambert-Balay *et al.*, 2008), Tunisia (Sdiri-Loulizi *et al.*, 2008), Hungary (Reuter *et al.*, 2009), China (Yang *et al.*, 2009) and Finland (Kaikkonen *et al.*, 2010). The P1 region sequences of AiV-1 were divided into two lineages, I and II, on the basis of their sequence distances and groupings (Pham *et al.*, 2008). The two sequences (those with underlined accession numbers in Figure 7.1b) previously identified from Vietnamese children (Pham *et al.*, 2008) clustered in lineage I while the sequence recovered in this study grouped in lineage II.

EVs may cause a wide spectrum of symptoms, from asymptomatic to serious or even life-threatening illness in some cases (Bessaud *et al.*, 2008) and the pathogenicity varies between types. In this study, EVs were detected in 8 samples from patients with

flu-like symptoms or fever or diarrhoea and pharyngitis (Table 7.2) – all symptoms that can be caused by EVs and pathogens other than picornaviruses (Kociolek & Shulman, 2012; Patel & Thillainayagam, 2009). From those samples, 4 EV types (E9, CVB4, CVA5, EV71) were identified. As non-picornavirus pathogens have not been determined from these samples, whether the identified EVs as well as AiV-1 were the causative agents of these symptoms in the infected patients was uncertain.

#### **7.4.3. Zoonotic origin of picornaviruses**

People in this study were at high risk of zoonotic infections due to frequent and close occupational contact with animals, especially slaughterhouse workers, animal health workers. Meanwhile, high infection frequencies of EVs, kobuviruses in pigs, cardioviruses, kobuviruses and HuVs in rats, and kobuviruses in civets were demonstrated in Chapter 3. However, no identified animal picornaviruses (Chapters 4 & 6) were detected in human samples. This suggests that these animals may not be the sources of picornavirus infections in humans in Vietnam.

In a broader view, studies have only shown rare detection of human EVs in a bat and some rats (Gregorio *et al.*, 1972), pigs (Grew *et al.*, 1970; Lomakina *et al.*, 2015), chickens (Graves & Oppenheimer, 1975), and NHPs as the most frequently reported source of zoonotic EVs (Harvala *et al.*, 2011b; Oberste *et al.*, 2013a; Oberste *et al.*, 2013b; Sadeuh-Mba *et al.*, 2014) and HPeVs (Oberste *et al.*, 2013b; Shan *et al.*, 2010). In other words, among the targeted picornaviruses, those reported with zoonotic potential are restricted to viruses of genera *Enterovirus* and *Parechovirus* in a limited number of hosts to date.

#### **7.4.4. Limitations of this study**

A limitation of this study was the small sample size (250 human samples screened). A large sample size is particularly important to obtain sufficient data for such viruses with low prevalence (from 0 – 3.2%) in the studied human populations as cosaviruses, parechoviruses, kobuviruses, cardioviruses and EVs. For this reason, it is apparent that a much larger sample size is required to capture most of the targeted picornaviruses circulating in Vietnam. From that, there will be more opportunities to detect rare

zoonotic events. This is especially necessary providing that only few studies have been performed on picornaviruses in Vietnam. Most previous picornavirus surveillances focused on EV71 and CVA16 as causes of HFMD (Geoghegan *et al.*, 2015; Khanh *et al.*, 2012; Phan *et al.*, 2007) or just detected EVs without further genetic characterization (Tan *et al.*, 2010; Tan *et al.*, 2014; Taylor *et al.*, 2012). A recent survey (unpublished) within the VIZIONS project on the same targeted picornaviruses in 683 hospitalised diarrhoeic patients, mostly children, identified 20 human EV types in species *EV-A* to *-C*, including CVA24 (the most common type) and EV71 – two types also reported from NHPs (Sadeuh-Mba *et al.*, 2014).

Moreover, infections with the targeted picornaviruses are transient. Studies showed the shedding durations in stool of EV and HPeV are up to 11 weeks (Chung *et al.*, 2001) and 93 days (Kolehmainen *et al.*, 2012), respectively. Although PCR is a quick and sensitive method for detection of pathogens, it is not suitable for samples collected outside of the detectable shedding period. This is supported by the survey of 683 hospitalised diarrhoeic patients, mostly children younger than 2 years of age, mentioned above. All children had received oral PV vaccine but PV vaccine strains were detected in only 3 patients. In contrast, specific antibodies against these picornaviruses normally persist for years or decades (Dotzauer & Kraemer, 2012). Serology testing for antibodies would provide a better way to measure total exposure. However, detection of antibodies confirms infections rather than GI carriage.

In summary, this chapter characterizes picornaviruses in human positive samples. In common with most previous studies, the results showed a clear distinction between the identified human viruses and animal viruses detected in the previous chapters as well as documented in literature. It indicates that no zoonotic events by the screened picornaviruses were detected or may have occurred in the studied populations during the course of this work.



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## Chapter 8. Concluding remarks

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The study was carried out to investigate the existence, genetic diversity, disease associations and potential animal sources of picornavirus infections in humans. Its main hypotheses are the targeted picornaviruses may have a contribution to diseases in humans and animals, and animals are infected with zoonotic picornaviruses in Vietnam. These were where knowledge gaps existed in the general literature and specifically in the context of Vietnam. The study aimed to answer the following questions:

- 1) *How prevalent and diverse are picornaviruses in studied samples and in comparison with other regions of the world?*
- 2) *Are the identified viruses associated with disease and age of infected humans and animals?*
- 3) *Which viruses are circulating among different animal species and geographic-matched human populations?*
- 4) *Which animals may be the sources of picornavirus infections in humans?*

The main empirical findings are chapter specific, with prevalence of picornaviruses in screened samples presented in Chapter 3; and subsequent characterization of picornaviruses in the positive samples from domestic pigs and boars, monkeys, rodents and civets, and human samples in Chapters 4 – 7. This chapter will synthesize the main findings to answer the four research questions.

### 8.1. Prevalence of picornaviruses in screened samples

More than 2,000 faecal samples from humans and a wide range of animals were screened for six picornavirus genera. In accordance with previous studies, the current work showed the existence of EV and kobuvirus in pigs and farm-bred boars (with frequencies of 8.9 – 79%); a high infection frequency of EV in monkeys from Cameroon (96.5%), cardiovirus in rats (6.6%), kobuvirus, cardiovirus and EV RNA detectable in human samples at low frequencies (0.4 – 3.2%), and absence of the screened picornaviruses in bats, chickens, ducks and shrews. Novel findings were

detection of kobuvirus in civets (16.7%) and a porcupine (1.6%); cardiovirus in porcupines (4.9%); kobuvirus and HuV in a wider range of rodent species than previously documented (Chapter 3).

## **8.2. Association of the identified viruses with disease and age of infected humans and animals**

Among the screened animals, clinical information and age data were available from pigs. In common with previous studies (Chen *et al.*, 2013; Park *et al.*, 2010), the current work showed a clear correlation of PKV but not EV infection with diarrhoea. However, the association was only observed in matured pigs (> 1 year of age). The role of PKV remains to be determined as there were no significant differences in its viral loads and VP1 sequences between groups of diarrhoeic and healthy pigs.

EV infection frequencies were highest in young pigs and generally decreased with pig age. This may reflect the role of acquired immunity in older pigs. For most EVs, immunity to reinfection is believed to be lifelong (Pepper *et al.*, 2011), as is protection provided by live PV vaccination (Doan *et al.*, 2012). On the contrary, infection frequencies of kobuvirus were not clearly correlated with age, with high prevalence in the youngest pigs, significantly lower prevalence in weaner to gilts but most common in matured diarrhoeic pigs. Rather than playing a direct causative role, it is also possible that diarrhoea secondary to infection with other pathogens may alter gut mucosal immunity and induce inflammatory processes that reactivate and increase the excretion of kobuviruses harboured in the GI tract to detectable levels.

In contrast to pigs, the lack of clinical information of other studied subjects (other animals) or screening for other non-picornavirus pathogens (human samples) prevented any conclusion on the association of other picornaviruses with disease and age of infected humans and animals.

## **8.3. Diversity of the identified viruses**

Viruses in the positive samples were characterized by sequencing of the VP4/VP2 or VP1 region. Application of VP1 type assignment on the basis of sequence distances

provides a quick and consistent method to identify the genetic diversity of EVs. Characterization of EVs infecting Vietnamese pigs and farm-bred boars showed high genetic diversity with 14 of 16 types in species *EV-G* to date, nine of which were novel (EV-G8 to -G16). Detection of the novel types demonstrates incomplete characterization of this virus species. Inter-type recombination was observed between EV-G8 and -G9 genome sequences (Chapter 4). Some EVs detected in monkeys belonged to species *EV-B* (SA5, EV-B110), *EV-H* (EV-H1) but the majority were unassigned EVs. Analysis of complete genome sequences of these novel EVs revealed recombination with a breakpoint locating at the border of P1 and P2. The hybrid nature of the new variants warrants the need to re-examine criteria for assignment of EVs into species (Chapter 5). Meanwhile, EVs infecting humans were identified as variants of E9, CV-B4 (members of *EV-B*) and EV-71, CV-A5 (*EV-A*) (Chapter 7).

For other picornaviruses, despite the lack of studies for VP1 sequence distance thresholds as alternatives for identification of serotypes, distances and phylogenetic analyses of VP4/VP2 or VP1 sequences helped demarcate their groupings. The identified kobuviruses included PKV (*Aichivirus C*) in pigs and farm-bred boars (Chapter 4), viruses in rodents and a civet clustering in species *Aichivirus A* (Chapter 6), and AiV-1 (*Aichivirus A*) in a human sample (Chapter 7). Rodent derived cardioviruses grouped with TRV, EMCV and Boone cardiovirus (Chapter 6) while cardiovirus in a human sample was identified as SAFV (Chapter 7). HuVs from rodents were highly divergent from the known HuVs and may represent novel types in species *Hunnivirus A* (Chapter 6).

#### **8.4. Absence of overlaps between viruses circulating among humans and other animals**

Cross-host exposures are an essential pre-condition for virus transfer to new hosts (Pike *et al.*, 2010; Parrish *et al.*, 2008). However, in this work, in spite of high infection frequencies of picornaviruses in animals and frequent contact with those animals of the studied human cohort, no overlaps at the type level defined by the ICTV Picornavirus Study Group were observed between picornaviruses detected in human and animal samples in Vietnam.

Therefore, the findings mentioned above reaffirm that the process of cross-species transmission is also driven by many other factors. In order to switch to a new host, a virus must have the ability to efficiently infect the appropriate cells of the new host. This process can be constrained by multiple host barriers, at different steps and levels such as binding to receptors, entry or fusion, trafficking within the cell, genome replication, and gene expression. The evolutionary relatedness of the hosts may also play a role in host switching. Viral host switches have occurred between closely or distantly related hosts as demonstrated by HIV establishment in humans from chimpanzees and transmission of avian influenza to humans. Currently, it appears impossible to predict the susceptibility of a new host. Each of these host barriers would require corresponding changes in the virus, resulting in difficulties for the virus to overcome the species barriers (Parrish *et al.*, 2008).

The non-overlaps between human and animal viruses in this thesis indicate that the screened animals may not be a source of picornavirus infections in humans. This is consistent with limited despite growing evidence of zoonotic transmission of picornaviruses to date. Among the screened viruses, only a small number of EV and HPeV serotypes with zoonotic potential have been reported from a rabbit, a fox, a bat, rats, dogs, chickens and NHPs with unknown transmission direction (Harvala *et al.*, 2011b), while SVDV evolved from CVB5 in pigs in the recent decades. CVB4 and CVA20 are the known reverse zoonotic picornaviruses which have switched host from humans to pigs (Brown *et al.*, 1973; Grew *et al.*, 1970; Knowles & McCauley, 1997; Lomakina *et al.*, 2015; Seechurn *et al.*, 1990).

The absence of zoonotic events presented here also has an impact on the current knowledge of documented zoonoses. In a review (Woolhouse and Gowtage-Sequeria, 2005), viruses were regarded as zoonotic or not at the species level. This work shows that at more detailed levels (e.g. type), human and animal viruses, although belonging to the same species, are clearly distinct and may have no zoonotic potential. Moreover, human pathogens have undergone major reclassification, such as EV68 and EV70, which are now assigned to the same species (*EV-D*) but which were treated as two separate species in the review (Woolhouse and Gowtage-Sequeria, 2005). With recent detection of human viruses in animals (e.g. EVs and HPeVs in NHPs), the zoonotic

status of species has now changed as well (such as *EV-A* to *-D*). These highlight the need to re-evaluate the actual number of virus species claimed to be zoonotic (816 [58%] of 1,407 human pathogen species) in that review.

### **8.5. Future directions**

In this thesis, RNA of viruses in species *EV-G* was detected in 79% of pig samples, of which *EV-G1* and *-G6* were the most common. However, no human samples from the high risk cohort populations were positive for these porcine EVs. This indicates *EV-G* viruses either have no zoonotic potential or rarely infect humans or their infections did occur in the studied cohort but in a short time and the acute infections were cleared at the time of sampling. PCR screening of a larger number (thousands) of human samples will provide a better understanding of their zoonotic potential. Alternatively, development of specific serological assays for seroprevalence studies of *EV-G1* and *-G6* would enable a better estimate of total human exposure to be made. These *EV-G* viruses may be similar to *LV*, which studies (Jääskeläinen *et al.*, 2013b; Niklasson *et al.*, 1999) have shown high seroprevalence in patient serum samples while no *LV* RNA has been detectable in human samples to date.

Furthermore, NHPs are the most frequently documented source of EVs and HPeVs with zoonotic potential to date. It is possible that for picornaviruses, the close genetic relationships between humans and NHPs support the transmission of picornaviruses between these two groups. In this thesis, only NHPs from remote areas with minimal human contact were screened for EVs. The identified EVs were separate from human EVs. More NHPs, particularly those with frequent human contact should be included in future studies. In Ho Chi Minh City, Vietnam, there is a tourist attraction site called Can Gio Monkey Island, which houses a population of about 1,500 long-tailed monkeys (*Macaca fascicularis*) in the natural condition where tourists can come in close proximity to feed and touch them. The large population of monkeys and their close contact with humans can facilitate the exchange and maintenance of picornaviruses and other viruses between the two groups. This monkey population may be a suitable sampling site for picornavirus studies in the future.

## **8.6. Conclusion**

In conclusion, the work undertaken within this PhD has focused on the existence, genetic characterization, zoonotic events and contribution to disease in studied humans and animals of picornaviruses. In addition to detection of known viruses, some novel variants of picornaviruses (EVs in pigs, boars and monkeys; cardioviruses, kobuviruses and HuVs in rodents) were discovered. The detected viruses show high genetic diversity, possibly no zoonotic potential, and evidence for recombination between EV genomes. Among these, only infection with kobuvirus was associated with diarrhoea in pigs while the contribution of viruses present in samples to patients' clinical status was inconclusive. Larger scale screening of human and other animal samples (e.g. NHPs in Vietnam) by PCR and serology methods will provide a better understanding of the possible animal sources and the role of picornavirus infections in human disease.

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## Appendix 2. Sequences obtained in Chapter 7

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